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The role of Sdf-1 in the migration and differentiation of stem cells during skeletal muscle regeneration.

Rozprawa doktorska
w zakresie nauk biologicznych
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Praca wykonana pod kierunkiem
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"Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration"

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"Stem cells migration during skeletal muscle regeneration - the role of Sdf-1/Cxcr4 and Sdf-1/Cxcr7 axis"

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Streszczenie

Przedstawiona praca doktorska dotyczy mobilizacji komórek macierzystych do uszkodzonej tkanki mięśniowej. Opisane w niej badania koncentrowały się na opracowaniu metod prowadzących do zwiększenia zdolności zarówno endogennych jak i egzogennych, tj. przeszczepianych komórek macierzystych do udziału w regeneracji mięśni szkieletowych myszy. Dotyczyły także poznania mechanizmów molekularnych, jakie towarzyszą zarówno migracji jak i różnicowaniu tych komórek.

Mięśnie szkieletowe, obok tkanek budujących skórę, są jedną z tkanek najbardziej narażonych na urazy. Z tego powodu ich prawidłowa regeneracja jest niezmiernie istotna dla prawidłowego funkcjonowania organizmu. Mięśnie szkieletowe składają się z wielojądrzastych włókien mięśniowych, które mają zdolność do kurczenia się, co stanowi podstawę ich funkcjonowania. W odpowiedzi na uraz uszkodzone włókna mięśniowe zostają usunięte przez napływające makrofagi, a następnie zastąpione przez nowe włókna [1]. Nowe włókna mięśniowe powstają dzięki fuzji jednojądrzastych komórek, które tworzą najpierw wielojądrzaste miotuby, „dojrzewające" następnie we włókna mięśniowe posiadające setki jąder komórkowych. W przypadku regeneracji mięśni kluczowe są więc komórki progenitorowe zdolne do różnicowania miogenicznego i fuzji, a co za tym idzie odtwarzania nowych włókien mięśniowych. W warunkach fizjologicznych pulę takich komórek tworzą komórki satelitowe, czyli komórki macierzyste mięśni szkieletowych, z których w wyniku podziałów komórkowych powstają zdolne do fuzji mioblasty [2]. Zapewniają one bardzo wydajną regenerację. Jednak w przypadku rozległych uszkodzeń, procesu starzenia, cukrzycy, nowotworów, a także chorób mięśniowych, np. dystrofii, naprawa tkanki nie przebiega prawidłowo. Konsekwencją nieprawidłowej regeneracji może być utrata funkcjonalności mięśni i ich zwiększona podatność na ponowne uszkodzenia [3]. Jedną z potencjalnych form wspomagania regeneracji mięśni szkieletowych mogłaby być terapia komórkowa polegająca na mobilizacji własnych komórek macierzystych lub na podawaniu pacjentowi egzogennych komórek macierzystych - izolowanych z tkanek pacjenta lub dawcy [4]. Komórki takie powinny mieć zdolność do uczestniczenia w odtwarzaniu włókien mięśniowych. Jedną z największych przeszkód w skutecznym zastosowaniu komórek macierzystych we wspomaganiu regeneracji mięśni szkieletowych jest ograniczona zdolność tych komórek do migracji w obrębie uszkodzonego mięśnia. Podanie komórek, które nie są zdolne do migracji skutkuje ich wyłącznie lokalnym udziałem w rekonstrukcji, a to nie wpływa na poprawę funkcjonowania mięśnia [5, 6].

Istnieje wiele populacji komórek macierzystych, które mogłyby zostać wykorzystane do wspomagania regeneracji. W prowadzonych przeze mnie badaniach, analizowałem trzy rodzaje takich komórek - pluripotencjalne zarodkowe komórki macierzyste (ESC, ang. Embryonic Stem Cells), multipotencjalne komórki macierzyste szpiku kostnego (BMSC, ang. Bone Marrow Stem Cells) oraz komórki satelitowe, czyli unipotencjalne komórki macierzyste mięśni szkieletowych. Wybór ten był podyktowany różnicami w potencjale miogenicznym tych komórek, czyli możliwością różnicowania w mioblasty i włókna mięśniowe, a także różnicami w ich zdolności do migracji i zasiedlania uszkodzonych mięśni szkieletowych [7, 8, 9].

W moich badaniach analizowałem rolę cytokiny Sdf-1 (ang. Stromal derived factor - 1) w mobilizacji komórek macierzystych zdolnych do uczestniczenia w regeneracji mięśni szkieletowych. W warunkach fizjologicznych cytokina ta jest produkowana w tkance w odpowiedzi na hipoksję, czyli obniżone stężenie tlenu, będące konsekwencją uszkodzenia. Jest ona chemoatraktantem dla komórek mających na swojej powierzchni receptor Cxcr4

(Cxcr4+). Receptor ten eksprymują komórki układu odpornościowego oraz wiele typów komórek macierzystych [10]. W prowadzonych badaniach postawiłem hipotezę, że mobilizowane przez Sdf-1 do regenerującego mięśnia endogenne i egzogenne komórki macierzyste mogą uczestniczyć w rekonstrukcji uszkodzonej tkanki.

Aby zweryfikować powyższą hipotezę analizowałem regenerację mięśni myszy pozbawionych funkcjonalnych alleli genu Pax7 (*Pax7-/-*). Myszy *Pax7-/-* charakteryzuje złożony fenotyp, jednak z punktu widzenia prowadzonych przeze mnie doświadczeń najistotniejsze jest to, że ich mięśnie cechuje prawie całkowity brak komórek satelitowych. Dzięki temu mogłem analizować regenerację zachodzącą przy istotnym niedoborze komórek, które w warunkach fizjologicznych odpowiedzialne są za odbudowę tej tkanki. W trakcie prowadzonych doświadczeń podawałem myszom podskórnie cytokinę G-CSF (ang. Granulocyte Colony Stymulating Factor) powodującą uwolnienie ze szpiku kostnego do krwioobiegu komórek macierzystych. Następnie uszkadzałem mięśnie szkieletowe, do których po dobie podawałem roztwór chemokiny Sdf-1, która stymuluje napływ komórek z receptorem Cxcr4, m.in. komórek macierzystych szpiku kostnego. Wykazałem, że Sdf-1 mobilizuje do uszkodzonego mięśnia komórki Cxcr4+ syntetyzujące dodatkowo marker CD34, a więc komórki macierzyste ze szpiku kostnego. Wykazałem, że efektem podawania Sdf-1 i mobilizacji komórek jest wzrost masy zrekonstruowanego mięśnia z jednoczesnym zmniejszeniem stopnia jego zwłóknienia - czyli ograniczenie powstawania tkanki łącznej. Wyniki te udowodniły, że zastosowanie G-CSF i Sdf-1 może stanowić skuteczny sposób na wspomaganie regeneracji mięśni szkieletowych w przypadkach niedoboru komórek satelitowych.

Fuzja komórek jest obok migracji kluczowym procesem zachodzącym podczas regeneracji mięśni szkieletowych. Przebiega ona z udziałem transbłonowych białek adhezyjnych. Postawiłem hipotezę, że zmiany w poziomie tych białek mogą przekładać się na zdolność komórek macierzystych do migracji i fuzji z mioblastami. Wykazałem, że Sdf-1 zwiększa poziom białka adhezyjnego CD9 w ESC i BMSC. Udowodniłem również, że mechanizm wzrostu poziomu CD9 w odpowiedzi na Sdf-1 jest zależny od pobudzenia receptora Cxcr4. Aby sprawdzić, czy wzrost poziomu CD9 wpływa na zdolność do fuzji badanych komórek z komórkami mięśniowymi, przeprowadziłem hodowlę mieszaną, w trakcie której hodowałem mioblasty z badanymi komórkami tj. BMSC bądź ESC. Analizowałem komórki kontrolne oraz takie, które uprzednio traktowałem Sdf-1. Mioblasty fuzjując ze sobą tworzą wielojądrowe miotuby. Jeżeli badane komórki mają zdolność do fuzji to wspólnie z mioblastami będą tworzyć hybrydowe miotuby. Częstość, z jaką powstają takie miotuby odzwierciedla potencjał badanych komórek do uczestniczenia w regeneracji mięśni szkieletowych. Wykazałem, że Sdf-1 zwiększa zdolność BMSC do uczestniczenia w tworzeniu hybrydowych miotub. Analiza ESC wykazała natomiast, że komórki te mają bardzo ograniczoną zdolność do tworzenia hybrydowych miotub, jednak stymulacja Sdf-1 wpływa na ich zdolność migracji. Pod wpływem Sdf-1 migrują one lepiej w hodowli i zamiast formować agregaty (jak ma to miejsce w hodowli kontrolnej) lokalizują się wzdłuż miotub powstających z mioblastów.

Wiedząc, że Sdf-1 wpływa na komórki macierzyste zarówno *in vivo* jak i *in vitro* stymulując ich migrację i fuzję postawiłem hipotezę, że Sdf-1 aktywuje wewnątrzkomórkowe ścieżki sygnalizacyjne związane z tymi procesami. Badałem również rolę receptorów dla Sdf-1, czyli Cxcr4 i Cxcr7 w omówionych procesach. W tym celu hodowałem *in vitro* mysie ESC, ludzkie mezenchymalne komórki macierzyste izolowane z galarety Whartona (MSC,

ang. Mesenchymal Stem Cells) i mioblasty pierwotne uzyskane z komórek satelitowych izolowanych z mięśni szkieletowych myszy. Aby sprawdzić, który z receptorów: Cxcr4 czy Cxcr7, jest zaangażowany w odpowiedź komórki na traktowanie Sdf-1 wykorzystałem technikę siRNA pozwalającą wyciszyć ekspresję badanych receptorów. Wykazałem, że zarówno ESC, MSC jak i mioblasty pierwotne migrują w gradiencie Sdf-1 w sposób zależny wyłącznie od receptora Cxcr4. Ponadto stymulacja Sdf-1 powoduje wzrost aktywności GTPaz Rac-1 oraz Cdc42, a więc czynników odpowiedzialnych za rearanżację cytoszkieletu aktynowego uczestniczącego w migracji komórek. Efekt ten jest również zależny wyłącznie od receptora Cxcr4. Wykazałem także, że stymulacja badanych komórek za pośrednictwem zarówno receptora Cxcr4 jak i Cxcr7 powoduje wzrost poziomu ufosforylowanej, a więc aktywnej, formy kinazy płytek przylegania (FAK, ang. Focal Adhesion Kinase). Aktywna forma FAK jest odpowiedzialna za tworzenie płytek przylegania, struktur niezbędnych w trakcie migracji komórek [Figura 1]. Analiza wykonana techniką mikromacierzy pozwoliła mi na określenie zmian w transkryptomie ESC: kontrolnych, stymulowanych Sdf-1 oraz poddanych wyciszeniu ekspresji receptorów Cxcr4 bądź Cxcr7. Wykazałem, że w ESC przekazywanie sygnału z udziałem receptora Cxcr4 wpływa na ekspresję i aktywność białek związanych przede wszystkim z migracją i adhezją, podczas gdy Cxcr7 reguluje ekspresję białek cytoszkieletowych (np. aktyny).

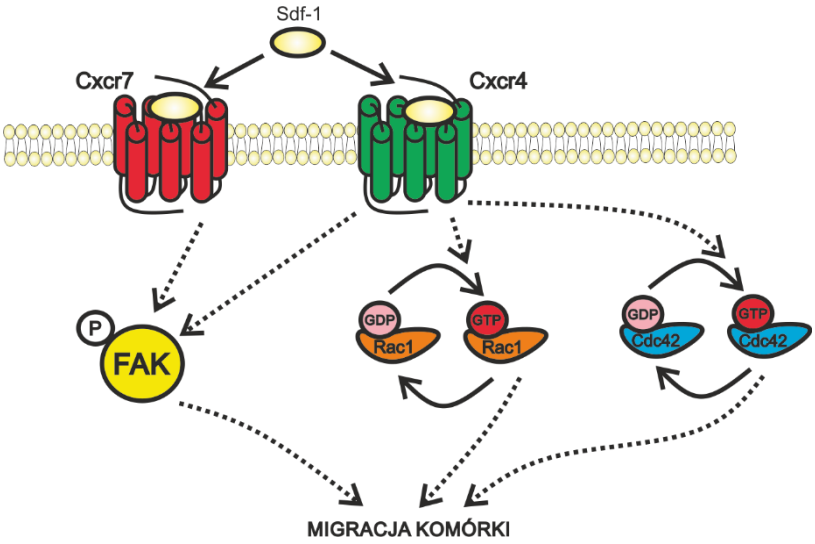

Figura 1. Sdf-1 aktywuje migrację komórek poprzez:

Zwiększenie aktywności GTPaz Rac1 oraz Cdc42. Jest to mechanizm zależny od receptora Cxcr4.

Sdf-1 działając poprzez receptory Cxcr4 oraz Cxcr7 zwiększa również aktywność kinazy płytek przylegania (FAK).

W kolejnym etapie doświadczeń określiłem jak zmiany indukowane przez Sdf-1 wpływają na zdolność komórek do zasiedlania uszkodzonych mięśni i udział w ich rekonstrukcji. Mioblasty pierwotne oraz ESC stymulowane Sdf-1 i/lub takie, w których wyciszyłem ekspresję receptora Cxcr4 lub Cxcr7, przeszczepiałem do regenerującego mięśnia szkieletowego. Wykazałem, że ESC mają bardzo ograniczoną zdolność do zasiedlania mięśnia, jednak stymulacja Sdf-1 powoduje wydajniejszą migrację tych komórek wzdłuż włókien mięśniowych. Mioblasty podane do regenerującego mięśnia są zdolne do uczestniczenia w regeneracji, stymulacja Sdf-1 zwiększa tę zdolność, podczas gdy wyciszenie ekspresji receptora Cxcr4 istotnie ją ogranicza. Obniżony poziom receptora Cxcr7 nie wpływa na zdolność mioblastów do zasiedlania regenerującego mięśnia.

Podsumowując, wyniki dotychczas opublikowanych badań wskazują na możliwość potencjalnego wykorzystania Sdf-1 do wspomagania regeneracji mięśni szkieletowych, zarówno w przypadku mobilizacji komórek endogennych, jak i po przeszczepianiu komórek. Mechanizm działania Sdf-1 opiera się przede wszystkim na stymulacji migracji komórek, co wykazałem zarówno w układzie *in vitro* jak i *in vivo*. Po drugie Sdf-1 promuje fuzję komórek macierzystych z komórkami mięśni szkieletowych za pośrednictwem białka adhezyjnego CD9. Zatem, podanie Sdf-1 do regenerującego mięśnia szkieletowego poprawia jego regenerację, głównie za sprawą zwiększenia udziału komórek macierzystych w odtwarzaniu nowych włókien mięśniowych [Figura 2]

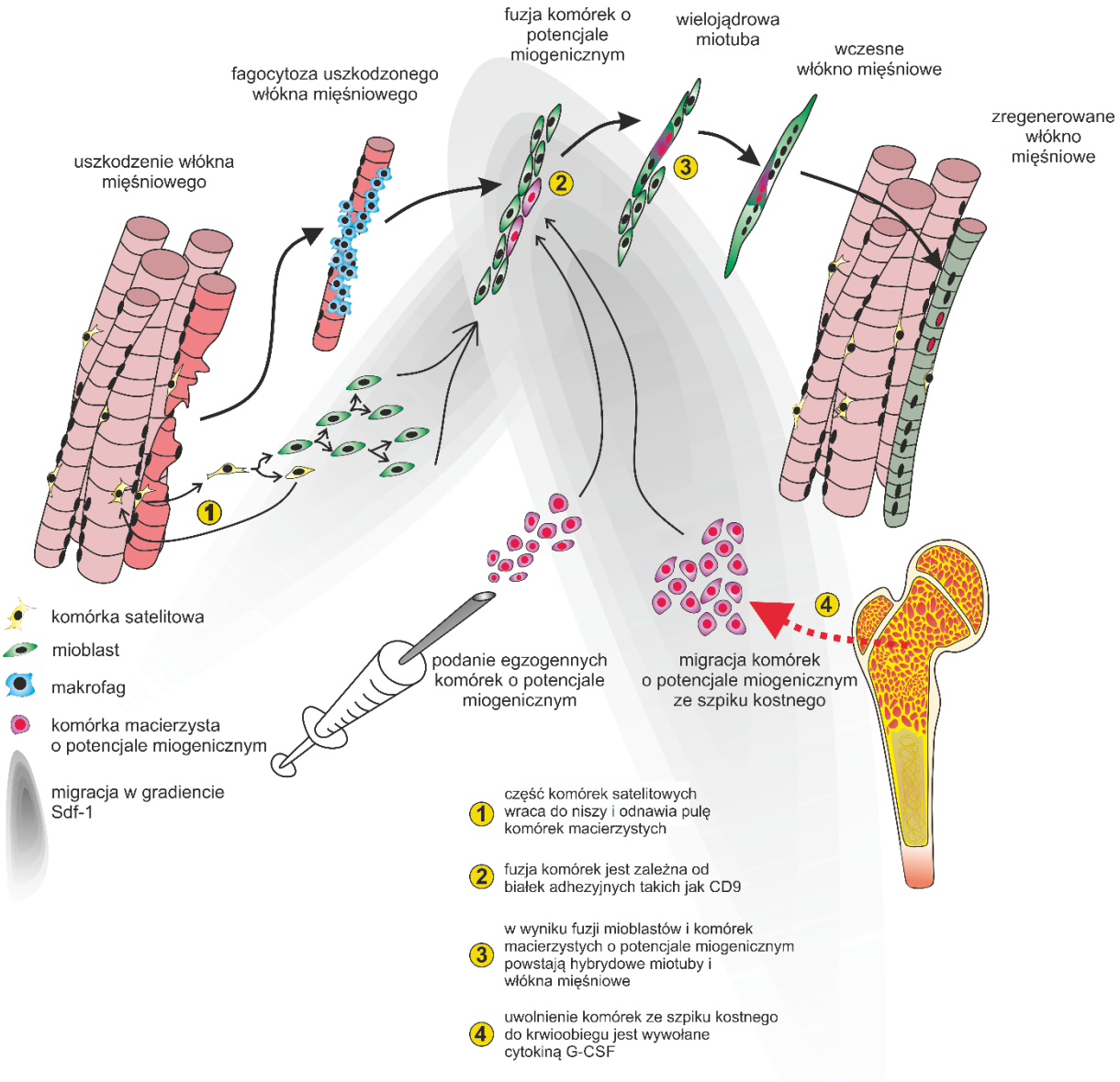

Figura 2. Regeneracja mięśni szkieletowych. Poza komórkami satelitowymi, z których powstają mioblasty, również inne endogenne i egzogenne komórki macierzyste wykazują zdolność do uczestniczenia w regeneracji mięśnia. Chemokina Sdf-1 tworzy gradient, który mobilizuje komórki macierzyste do regenerującego mięśnia, przez co zwiększa ich zdolność do współtworzenia nowych włókien mięśniowych.

1. Rigamonti, E., et al., *Macrophage plasticity in skeletal muscle repair.* Biomed Res Int, 2014. **2014**: p. 560629.
2. Relaix, F. and P.S. Zammit, *Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage.* Development, 2012. **139**(16): p. 2845-56.
3. Serrano, A.L., et al., *Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease.* Curr Top Dev Biol, 2011. **96**: p. 167-201.
4. Tedesco, F.S., et al., *Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells.* J Clin Invest, 2010. **120**(1): p. 11-9.
5. Price, F.D., K. Kuroda, and M.A. Rudnicki, *Stem cell based therapies to treat muscular dystrophy.* Biochim Biophys Acta, 2007. **1772**(2): p. 272-83.
6. Skuk, D., M. Goulet, and J.P. Tremblay, *Use of repeating dispensers to increase the efficiency of the intramuscular myogenic cell injection procedure.* Cell Transplant, 2006. **15**(7): p. 659-63.
7. Archacka, K., et al., *Competence of in vitro cultured mouse embryonic stem cells for myogenic differentiation and fusion with myoblasts.* Stem Cells Dev, 2014. **23**(20): p. 2455-68.
8. Grabowska, I., et al., *Restricted myogenic potential of mesenchymal stromal cells isolated from umbilical cord.* Cell Transplant, 2012. **21**(8): p. 1711-26.
9. Ferrari, G., et al., *Muscle regeneration by bone marrow-derived myogenic progenitors.* Science, 1998. **279**(5356): p. 1528-30.
10. Ceradini, D.J., et al., *Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1.* Nat Med, 2004. **10**(8): p. 858-64.

Summary

Presented PhD thesis concerns mobilization of stem cells into damaged skeletal muscle. Described experiments are focused on the development of methods improving the ability of endogenous and exogenous stem cells to participate in muscle regeneration. Moreover, described studies allowed to define the molecular pathways underlying cell migration and differentiation.

Skeletal muscle and skin tissues are among the ones that are frequently exposed to injury. Their regeneration must be efficient in order to secure proper functioning of organism. Skeletal muscles are composed of multinucleated fibers that are able to contract. In consequence of injury damaged fibers are removed by infiltrating macrophages and new fibers are formed to replace the damaged ones [1]. The new muscle fibers are formed by the cytoplasmic fusion of mononucleated precursor cells – myoblasts. Myoblasts fuse with each other to form multinucleated myotubes which mature to become fully functional muscle fibers containing hundreds of nuclei.

Thus, in case of skeletal muscle regeneration myoblasts able to fuse and then recreate muscle fibers are the key players in this process. Under physiological conditions these cells result from the differentiation of satellite cells, i.e. skeletal muscle stem cells. Satellite cells guarantee an efficient regeneration [2]. However, in consequence of extensive damage, aging or as a result of some pathological conditions regeneration may be not efficient. As a result muscle can lose functionality and become more prone to the next damage [3]. Cell therapy is among the approaches aiming to improve muscle regeneration. Such therapy could base on the administration of stem cells which are able to participate in muscle regeneration [4]. Among many complications associated with the transplantation of stem cells, which cause that the improvement of muscle regeneration is not sufficient, is poor migration ability of cells within the regenerating muscle. Such failure to migrate limits the regeneration only to the site of cells injection and thus has no impact on muscle function [5, 6].

The are many populations of stem cells which could be tested as a tool to improve skeletal muscle regeneration. I decided to test three types of those cells: pluripotent embryonic stem cells (ESCs), multipotent cells isolated from bone marrow (BMSCs) and unipotent satellite cells isolated from the skeletal muscles. Thus, I selected cells which represent different myogenic potential and different ability to migrate [7, 8, 9].

Next, I focused at the role of Sdf-1 (Stromal derived factor -1) in stem cells migration and participation in muscle regeneration. Under physiological conditions Sdf-1 is produced in the response to hypoxia, which is one of the symptoms of tissue injury. Sdf-1 mobilizes migration of cells that express Cxcr4 receptor on their surface [10]. Among such Cxcr4+ cells are immune cells and also many types of stem cells. The hypothesis I tested stated that Sdf-1 could mobilize stem cells that are characterized by the ability to undergo myogenic differentiation.

To examine abovementioned hypothesis I tested whether endogenous stem cells could be mobilized to regenerating muscle. If mobilized cells would be able to participate in the formation of new muscle fibers, muscle regeneration should be improved. In these experiments I took advantage of mice lacking functional Pax7 gene (*Pax7-/-*). *Pax7-/-* mice are characterized by complex phenotype but the most important was severely limited number of satellite cells in *Pax7-/-* mice muscles. Thanks to this I could analyze muscle regeneration

occurring in the absence of satellite cells. Importantly, I could also check if endogenous stem cells could replace lacking satellite cells. Thus, Pax7-/- mice were subcutaneously injected with Granulocyte Colony Stimulating Factor (G-CSF) which induces mobilization of stem cells from bone marrow to the bloodstream. Then skeletal muscle was injured and injected with Sdf-1 what stimulated the infiltration of Cxcr4 positive cells, i.e. BMSCs. Further analyzes proved that Sdf-1 mobilized Cxcr4+ and CD34+ cells to the site of the injury. In consequence skeletal muscle mass was increased and fibrosis was decreased. These results indicate that Sdf-1 together with G-CSF could be used as an effective way to improve skeletal muscle regeneration in case of satellite cells deficiency. G-CSF stimulates releasing of BMSC from bone marrow to the bloodstream then Sdf-1 acts as a chemoattractant stimulating stem cells migration to muscle.

Migration of the cells is the key "element" allowing proper muscle regeneration. However, it is the cell fusion which is the most important aspect of muscle repair. This process bases on adhesion proteins expressed by fusing cells. Thus, I examine if Sdf-1 will have an impact on the level of adhesion proteins in stem cells. Importantly, Sdf-1 stimulation led to the increase in the level of CD9 tetraspanin in ESCs and BMSCs. To check if CD9 up-regulation has an impact on cells fusion I co-cultured these stem cells with myoblasts. Such co-cultures will result in the formation of the hybrid myotubes only if analyzed cells (e.g. ESCs or BMSCs) present the ability to fuse with myoblast. Thus, hybrid myotubes contains nuclei of myoblast and examined cells. The frequency of hybrid myotubes formation reflects potency of examined stem cells to participate in muscle regeneration. Sdf-1 increased the ability of BMSCs to participate in the formation of hybrid myotubes. This effect was associated with the up-regulation of CD9 expression. ESCs analysis revealed that these cells have limited ability to fuse with myoblasts. However, Sdf-1 stimulated these cells to migrate and localize along forming myotubes. In control cultures they remained aggregated. Finally, I proved that the mechanism of CD9 up-regulation in response to Sdf-1 based on a Cxcr4 receptor.

Knowing that Sdf-1 influences stem cells both *in vivo* and *in vitro* and stimulates their migration and fusion I was decided to focus at intracellular pathways engaged in the migration. Simultaneously I studied which Sdf-1 receptors: Cxcr4 or Cxcr7 are involved in the regulation of cellular migration. Mouse ESCs, human MSCs and myoblasts derived from mouse satellite cells were examined in this study. Using siRNA technique I silenced expression of Cxcr4 or Cxcr7 receptor in these cells. Next, I stimulated the cells with Sdf-1. I have shown that both ESCs, MSCs, and myoblasts migrate in a gradient of Sdf-1 in Cxcr4 but not Cxcr7 dependent manner. Moreover, Sdf-1 increased the activity of GTPases Rac1 and Cdc42, i.e. the factors participating in migration by influencing the reconstruction of actin cytoskeleton. Similarly, this effect depended on the Cxcr4 but not Cxcr7 receptor. Sdf-1 also increased the level of active Focal Adhesive Kinase (FAK). However, this phenomenon was both Cxcr4 and Cxc7 dependent. FAK was also necessary for migration of tested cells [Figure 1]. Transcriptome analysis of control ESCs, those Sdf-1 treated, and those with Cxcr4 or Cxcr7 silenced showed that Cxcr4 regulates migration and adhesion pathways whereas Cxcr7 dependent signaling is important for expression of some cytoskeleton proteins (e.g. actin).

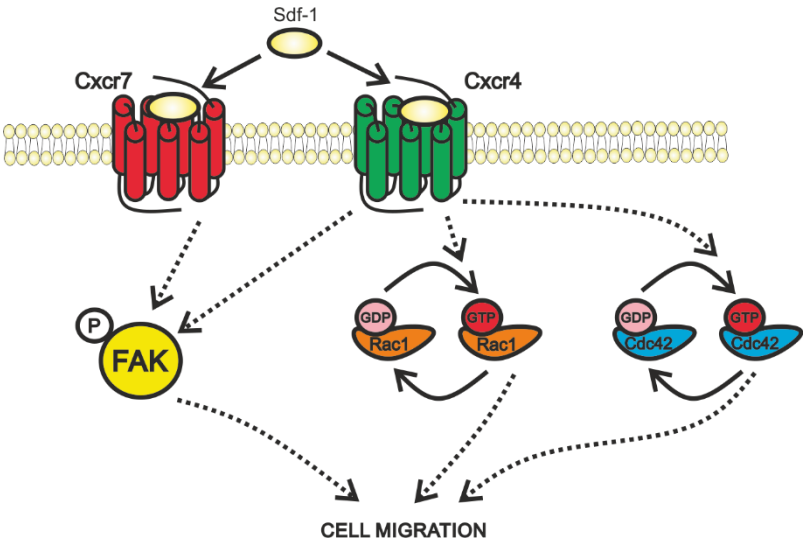

Figure 1. Sdf-1 stimulate migration of cell through:

Increased the activity of GTPase Rac1 and Cdc42. The effect is dependent on Cxcr4 receptor.

Sdf-1 increased the activity of Focal Adhesion Kinase (FAK) in Cxcr4 and Cxcr7 dependent manner.

Finally, primary myoblasts and ESCs that were stimulated with Sdf-1, as well as those ones in that either Cxcr4 or Cxcr7 expression was silenced, were transplanted to regenerating skeletal muscle. Next, the ability of this cells to participate in muscle regeneration were examined. ESCs presented the limited ability to home within the muscle and did not participate in the myofiber formation. However, similarly as it was observed in *in vitro* cultures Sdf-1 stimulated these cells to migrate within regenerating muscle and localize along fibers. In case of myoblasts Sdf-1 stimulation increased their ability to fuse. Silencing of Cxcr4 significantly decreased this ability. Cxcr7 was not involved in this process.

Summarizing, results of experiments that I performed during my PhD studies indicate that Sdf-1 could be used to improve skeletal muscle regeneration based on endogenous or exogenous stem cells. Sdf-1 not only stimulate migration but also cell fusion by increasing CD9 level. Both mechanism increase the ability of stem cells to participate in the reconstruction of new muscle fibers [Figure 2].

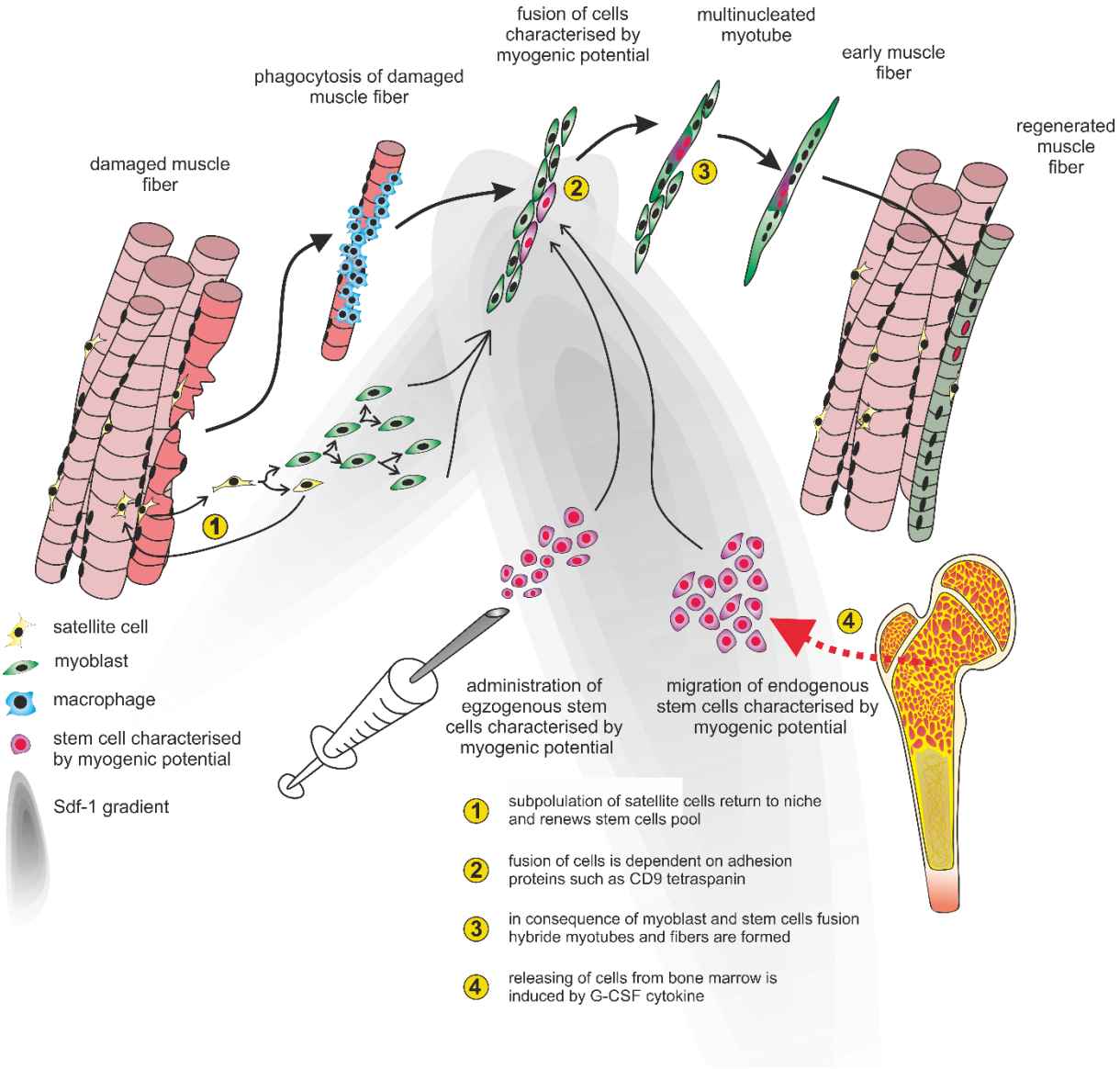

Figure 2. Skeletal muscle regeneration. Myoblast derived from satellite cells participate in skeletal muscle reconstruction, but also endogenous and egzogenous stem cells characterized by myogenic potential can take part in myotube and fiber formation. Sdf-1 chemokine gradient mobilize stem cells to the side of injury and by this increase ability to participate in new muscle fiber formation.

1. Rigamonti, E., et al., *Macrophage plasticity in skeletal muscle repair.* Biomed Res Int, 2014. **2014**: p. 560629.
2. Relaix, F. and P.S. Zammit, *Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage.* Development, 2012. **139**(16): p. 2845-56.
3. Serrano, A.L., et al., *Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease.* Curr Top Dev Biol, 2011. **96**: p. 167-201.
4. Tedesco, F.S., et al., *Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells.* J Clin Invest, 2010. **120**(1): p. 11-9.
5. Price, F.D., K. Kuroda, and M.A. Rudnicki, *Stem cell based therapies to treat muscular dystrophy.* Biochim Biophys Acta, 2007. **1772**(2): p. 272-83.
6. Skuk, D., M. Goulet, and J.P. Tremblay, *Use of repeating dispensers to increase the efficiency of the intramuscular myogenic cell injection procedure.* Cell Transplant, 2006. **15**(7): p. 659-63.
7. Archacka, K., et al., *Competence of in vitro cultured mouse embryonic stem cells for myogenic differentiation and fusion with myoblasts.* Stem Cells Dev, 2014. **23**(20): p. 2455-68.
8. Grabowska, I., et al., *Restricted myogenic potential of mesenchymal stromal cells isolated from umbilical cord.* Cell Transplant, 2012. **21**(8): p. 1711-26.
9. Ferrari, G., et al., *Muscle regeneration by bone marrow-derived myogenic progenitors.* Science, 1998. **279**(5356): p. 1528-30.
10. Ceradini, D.J., et al., *Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1.* Nat Med, 2004. **10**(8): p. 858-64.

Cel pracy

Celem prowadzonych przeze mnie doświadczeń było opracowanie metod wspomagania regeneracji mięśni szkieletowych ssaków. Badaną przeze mnie metodą była indukcja migracji komórek macierzystych, które potencjalnie mogłyby uczestniczyć w procesie odtwarzania włókien mięśniowych w rekonstruowanej tkance.

Postawiłem nadrzędną hipotezę, że chemokina Sdf-1 uczestniczy w mobilizacji komórek macierzystych do uszkodzonej tkanki. Napływające komórki są zdolne do wspomagania regeneracji mięśnia, co przekłada się na zwiększenie wydajności tworzenia nowych włókien mięśniowych. Weryfikowałem następujące hipotezy szczegółowe:

1. Sdf-1 mobilizuje endogenne komórki macierzyste ze szpiku kostnego do regenerującego mięśnia szkieletowego, co może wspomagać regenerację mięśnia w przypadku niewystarczającej puli komórek satelitowych.

2. Stymulacja komórek macierzystych za pomocą Sdf-1 powoduje zmiany w poziomie białek adhezyjnych zaangażowanych w migrację i fuzję komórek macierzystych. Zmiany w poziomie tych białek wpływają na wydajniejszą fuzję z komórkami mięśniowymi.

3. Sdf-1, działając przez receptory Cxcr4 i Cxcr7, aktywuje wewnątrzkomórkowe ścieżki sygnalizacyjne regulujące aktywność i ekspresję białek zaangażowanych w migracje i fuzję. Stymulacja przeszczepianych komórek macierzystych za pomocą Sdf-1 zwiększa ich zdolność do uczestniczenia w regeneracji mięśnia.

Omówienie wyników opisanych w publikacjach włączonych do rozprawy doktorskiej.

Mobilizacja endogennych komórek macierzystych ze szpiku kostnego do regenerującego mięśnia szkieletowego. Wykorzystanie cytokin Sdf-1 i G-CSF.

Publikacja oryginalna 1: *Sdf-1 and G-CSF treatment improves regeneration of Pax7-/- mice skeletal muscles:*

Kamil Kowalski, Rafał Archacki, Karolina Archacka, Władysława Stremińska, Anna Paciorek, Magdalena Gołąbek, Maria A. Ciemerych, Edyta Brzóska,

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W warunkach fizjologicznych mięśnie szkieletowe ssaków regenerują bardzo dobrze. Komórki satelitowe, czyli komórki macierzyste mięśni szkieletowych, po uszkodzeniu mięśnia różnicują w mioblasty, które fuzjując ze sobą odtwarzają zniszczone włókna mięśniowe. W określonych sytuacjach regeneracja może być jednak utrudniona, bądź też zupełnie nie zachodzić. W przypadku człowieka przykładami takich sytuacji są urazy np. u chorych na nowotwór (kacheksja), bądź u osób w podeszłym wieku (sarkopenia). U takich osób pula komórek satelitowych jest mniejsza, a same komórki są mniej funkcjonalne. Badanie metod wspomagania regeneracji mięśni szkieletowych z wykorzystaniem myszy, jako modelu badawczego, jest trudne gdyż mięśnie tych zwierząt regenerują bardzo wydajnie. Istnieją jednak szczepy myszy transgenicznych, które umożliwiają częściowe odwzorowanie sytuacji, gdy regeneracja mięśnia nie zachodzi prawidłowo. Jednym z takich modeli badawczych są myszy pozbawione funkcjonalnych alleli genu Pax7. Myszy *Pax7-/-* cechuje złożony fenotyp. Jednak z punktu widzenia analiz regeneracji mięśni szkieletowych najistotniejsze jest to, że ich mięśnie cechuje prawie całkowity brak komórek satelitowych. Wykorzystując w moich badaniach ten model mogłem analizować regenerację mięśni szkieletowych zachodzącą przy istotnym niedoborze komórek satelitowych, a wiec komórek które w warunkach fizjologicznych odpowiedzialne są za naprawę tej tkanki. Byłem więc w stanie "zasymulować" sytuację, gdy uszkodzenie zachodzi w mięśniu, w którym komórki satelitowe nie są wystarczająco liczne lub nie są funkcjonalne. Mogłem więc sprawdzić, czy możliwa jest regeneracja mięśnia bez udziału mioblastów powstałych z komórek satelitowych, w oparciu o populację innych komórek macierzystych, np. tych endogennych, obecnych np. w szpiku kostnym.

Największym wyzwaniem tego projektu była mobilizacja endogennych komórek macierzystych do regenerujących mięśni. Aby to osiągnąć wykorzystałem chemokinę Sdf-1, która kieruje migracją komórek powodując ich napływ zgodnie z jej rosnącym gradientem. Uszkodzony mięsień był nastrzykiwany roztworem Sdf-1, tak aby gradient tego czynnika był atraktantem dla komórek macierzystych obecnych w organizmie myszy *Pax7-/-*. Dodatkowo, w celu zwiększenia liczby komórek macierzystych, które mogą napłynąć do mięśnia szkieletowego, myszy były uprzednio stymulowane G-CSF (ang. Granulocyte - Colony Stimulating Factor), który powoduje uwolnienie komórek ze szpiku kostnego do krwioobiegu. W ten sposób powstał mysi model regeneracji mięśni szkieletowych zachodzącej bez udziału

komórek satelitowych, w oparciu o komórki macierzyste mobilizowane ze szpiku kostnego. Wstrzyknięcie Sdf-1 do regenerującego mięśnia powoduje, że napływa do niego większa liczba komórek syntetyzujących receptor Cxcr4 i marker CD34, a więc komórek macierzystych szpiku kostnego. Obecność tych komórek w mięśniu została zweryfikowana z wykorzystaniem analiz mRNA kodującego powyższe markery, jak i białka z zastosowaniem technik immunofluorescencyjnych, Western-blott oraz cytometrii przepływowej.

Konsekwencją obecności w mięśniu komórek napływających w odpowiedzi na Sdf-1 był zarówno wzrost masy mięśnia jak i poprawa jego regeneracji, co przejawiało się mniejszym stopniem zwłóknienia, przy jednocześnie lepszej architekturze mięśnia. Mięśnie nastrzyknięte roztworem Sdf-1 w myszach stymulowanych G-CSF cechował również większy odsetek włókien eksprymujących zarodkową izoformę ciężkich łańcuchów miozyny. Ekspresja tej izoformy miozyny świadczy o obecności nowopowstałych włókien, czyli o prawidłowym procesie regeneracji. Zaobserwowałem również, że włóknom mięśniowym wyizolowanym z mięśni nastrzykniętych Sdf-1 towarzyszyło więcej komórek, w porównaniu do włókien z mięśni myszy kontrolnych.

Zaprezentowane wyniki sugerują, że zastosowanie G-CSF i Sdf-1 może stanowić skuteczny sposób na wspomaganie regeneracji mięśni szkieletowych w przypadkach, gdy liczba komórek satelitowych nie jest wystarczająca. G-CSF powoduje mobilizację do krwioobiegu komórek potencjalnie mogących uczestniczyć w regeneracji, podczas gdy podany miejscowo Sdf-1 tworzy gradient stymulujący napływ tych komórek do uszkodzonej tkanki.

Publikacja oryginalna 1, *Sdf-1 and G-CSF treatment improves regeneration of Pax7-/- mice skeletal muscles:*

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Stromal derived factor-1 and granulocyte-colony stimulating factor treatment improves regeneration of *Pax7*−/− mice skeletal muscles

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Abstract

Background The skeletal muscle has the ability to regenerate after injury. This process is mediated mainly by the muscle specific stem cells, that is, satellite cells. In case of extensive damage or under pathological conditions, such as muscular dystrophy, the process of muscle reconstruction does not occur properly. The aim of our study was to test whether mobilized stem cells, other than satellite cells, could participate in skeletal muscle reconstruction.

Methods Experiments were performed on wild-type mice and mice lacking the functional *Pax7* gene, that is, characterized by the very limited satellite cell population. Gastrocnemius mice muscles were injured by cardiotoxin injection, and then the animals were treated by stromal derived factor-1 (Sdf-1) with or without granulocyte-colony stimulating factor (G-CSF) for 4 days. The muscles were subjected to thorough assessment of the tissue regeneration process using histological and *in vitro* methods, as well as evaluation of myogenic factors' expression at the transcript and protein levels.

Results Stromal derived factor-1 alone and Sdf-1 in combination with G-CSF significantly improved the regeneration of *Pax7*−/− skeletal muscles. The Sdf-1 and G-CSF treatment caused an increase in the number of mononucleated cells associated with muscle fibres. Further analysis showed that Sdf-1 and G-CSF treatment led to the rise in the number of CD34+ and Cxcr4+ cells and expression of Cxcr7.

Conclusions Stromal derived factor-1 and G-CSF stimulated regeneration of the skeletal muscles deficient in satellite cells. We suggest that mobilized CD34+, Cxcr4+, and Cxcr7+ cells can efficiently participate in the skeletal muscle reconstruction and compensate for the lack of satellite cells.

Keywords Differentiation; Myoblast; G-CSF; Regeneration; Sdf-1 (CXCL12); Skeletal muscle; Stem cells

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Introduction

Skeletal muscle fibres contain several hundred nuclei within a continuous cytoplasm. Fibres are accompanied by muscle specific stem cells, that is, satellite cells that are localized between the sarcolemma and the basal lamina. These cells are responsible for postnatal muscle growth and regeneration (reviewed in Relaix and Zammit[1]). Responding to muscle injury, satellite cells become activated, become myoblasts, and fuse to form myotubes and then new muscle fibres. Simultaneously, with the activation of satellite cells, cytokines and chemokines released locally upon injury attract inflammatory cells, which infiltrate injured muscle and remove damaged fibres. Neutrophils are the first population of leukocytes that appear within the injured muscle and participate in the removal of muscle debris as well as exacerbate satellite cell activation. Then, macrophages accumulate and phagocyte apoptotic and necrotic cells and fibres, and they mediate the remodelling of the extracellular matrix, the formation of new vessels, and activation of myogenic precursors (reviewed in Rigamonti *et al.*[2]).

The ability of satellite cells to reconstruct the damaged muscle is remarkable. For example, it has been demonstrated that satellite cells connected to a single muscle fibre can recreate the number of myoblasts equal to the number of nuclei forming the parent fibre within just 4 days following a muscle injury.[3] Moreover, satellite cells are capable of self-renewing their population in adult muscles, which was first postulated by Moss and Leblond.[4] The capacity of satellite cells to self-renew was experimentally proved by Collins and coworkers, who transplanted a single muscle fibre together with few satellite cells to an injured skeletal muscle that was depleted of endogenous satellite cells by irradiation.[5] As a result of such grafting, the functional muscle fibres were reconstructed, and also a few hundred new satellite cells were observed.[5] These results were confirmed by Sacco and coworkers, who showed that even a single satellite cell transplantation allowed the reconstruction of the injured muscle and the restoration of the satellite cell pool.[6] Importantly, these cells were functional, that is, could orchestrate further rounds of muscle regeneration. Therefore, satellite cells and myoblasts resulting from them have been considered as myogenic cells, which could be used in cell-based muscle therapy. The ability of transplanted myoblasts to participate in muscle regeneration and restoration of dystrophin expression was shown using dystrophin deficient mdx mice.[7] However, the results of clinical trials using myoblasts for human Duchenne muscular dystrophy were disappointing (e.g. Karpati *et al.*[8]). Major limitations in the application of satellite cells or myoblasts in the management of muscular dystrophy is the restricted ability of these cells to migrate through vasculature and effectively engraft the injured muscle, as well as the rapid cell death following transplantation, which could be overcome by immunosuppression (reviewed in Briggs and Morgan[9]). On the other hand, high-density injection protocols allow to avoid the issue of limited migration of transplanted cells, but are only applicable to easily accessible small muscle groups.[10] Apart from satellite cells, many other populations of muscle-derived stem cells, bone marrow, or peripheral blood cells manifest myogenic potential *in vitro* and *in vivo*. The mesoangioblasts,[11] pericytes,[12] and CD133+ cells[13] make up the most interesting cells from the therapeutic point of view. However, the majority of data on the use of various stem cells in skeletal muscle therapy refers to the transplantation of exogenous cells into the injured muscle. The functions of endogenous cells, other than satellite cells, during skeletal muscle regeneration remain largely obscure.

In the present study, we attempted to mobilize stem cells into the site of the injury using stromal derived factor-1 (Sdf-1) and to increase the number of mobilized stem cells using granulocyte-colony stimulating factor (G-CSF). Bone marrow is the main source of Sdf-1, but this cytokine is also expressed in a wide variety of organs, such as the heart, liver, spleen, kidney, and brain (reviewed in Cencioni *et al.*[14]). It is well established that Sdf-1 induces migration of different stem cell types, that is, satellite cells,[15] CD34+ haematopoietic progenitor cells,[16] or mesenchymal stem cells.[17] Sdf-1 mobilizes transplanted stem cells to tissues affected by ischemia as it was shown for limbs and hearts (e.g. Elmadbouh *et al.* and Kuliszewski *et al.*[18,19]). G-CSF is the other factor involved in the mobilization of stem cells from the bone marrow to peripheral blood (reviewed in Alvarez *et al.*[20]). G-CSF was shown to increase the level of cytokines in serum, reduce the numbers of bone marrow macrophages, inhibit the activity of osteoblasts, which are the main source of Sdf-1 in bone marrow, increase the number of osteoclasts that release cathepsin K, and activate the CD26 protease that degrades Sdf-1 (reviewed in Hoggatt and Pelus[21]). Thus, the main mechanism of G-CSF action relies on down-regulation of Sdf-1 level in the bone marrow. G-CSF and erythropoietin induce neovascularization of infracted rat myocardium, however, it is not clear whether it involves the mobilization of endothelial progenitor cells (EPCs). G-CSF also inhibits apoptosis of cardiomyocytes and reduces the development of fibrosis in heart muscle.[22]

To study the impact of Sdf-1 and G-CSF on stem cell mobilization to the injured muscle, we used mice lacking functional Pax7 transcription factor (*Pax7*−/−). These mice are characterized by a very limited satellite cell population[23] and cannot efficiently regenerate injured muscles.[24] Thus, the induction of skeletal muscle regeneration in *Pax7*−/− mice must be dependent on cells other than satellite cells. We aimed to verify whether the endogenous (not transplanted) stem cells, mobilized from bone marrow, could participate in muscle regeneration and compensate for the lack of satellite cells.

Materials and methods

Mice

All performed experiments were approved by the Local Ethical Commission No 1 in Warsaw—permission No. 186/2011.

The 7-day-old male or female F_1 (C57Bl6N x 129Sv) *Pax7*+/+ (*wt*) and F_1 (C57Bl6N x 129Sv) *Pax7*−/− mice were used. C57Bl6N *Pax7*+/− have been generated with the insertion of a sequence encoding the neomycin gene into the first exon of the paired box domain of one Pax7 locus according to the method described previously.[25] Such mutation leads to the abolishment of DNA-binding activity of the Pax7 protein. Next, C57Bl6N *Pax7*+/− mice were crossed with 129Sv mice, and F_1 (C57Bl6N x 129Sv) were generated.

Genotyping

Genomic DNA was isolated from the tails of 4- to 5-day-old mice. Briefly, tail tips were cut and heated for 10 min at 95°C in 50 mM NaOH. Then, 1 M Tris (pH 8) was added, and samples were centrifuged, 1 μL of supernatant was used for PCR.

Reactions were performed with RedTaq Ready Mix (Sigma-Aldrich, St. Louis, MO, USA) at the following conditions: 32 cycles of (30 s at 94°C, 30 s at 65°C, and 90 s at 72°C) preceded by 60 s at 94°C, and followed by 10 min at 72°C. Genotypes of *Pax7*−/− mice were confirmed by PCR reaction using the following primers:
[GGGCTTGCTGCCTCCGATATAGC, GTGGGGTCTTCATCAACGGTC, TCGTGCTTTACGGTATCGCCGCTCCCG]. Wild-type allele was represented by 200 kb product, while the *Pax7*- allele by 700 kb product.[26]

Muscle injury and treatments

Intact muscles: gastrocnemius muscles of 8-day-old F_1 (C57BI6N x 129Sv) Pax7+/+ (*wt*) or *Pax7*−/− male or female mice were injected with 10 ng/10 μL (*wt*) or 5 ng/5 μL (*Pax7*−/−) of Sdf-1 (Life Technologies, Van Allen Way Carlsbad, CA, USA) dissolved in 0.9% NaCl (saline). Contralateral muscles were injected with the same volume of saline. Next, 6 days after Sdf-1 treatment, *wt* and *Pax7*−/− mice were sacrificed and the muscles were isolated. Injured muscles: both gastrocnemius muscles of 7-day-old F_1 (C57BI6N x 129Sv) *Pax7* +/+ (*wt*) or *Pax7*−/− male or female mice were injected with 5 μL (*wt*) or 3 μL (*Pax7*−/−) of cardiotoxin (ctx, 0.2 mM, Sigma-Aldrich). Twenty four hours post injury muscles were injected with 10 ng/10 μL (*wt*) or 5 ng/5 μL (*Pax7*−/−) of Sdf-1. Contralateral muscles were injected with the same volume of saline. Next, 4 or 7 days after injury, mice were sacrificed and muscles were isolated. Additionally, 4 days after injury, peripheral blood of the animals was collected. Sdf-1 and G-CSF treatment: both gastrocnemius muscles of 7-day-old F1 (C57BI6N x 129Sv) *Pax7*+/+ (*wt*) or *Pax7*−/− male mice were injected with 5 μL (*wt*) or 3 μL (*Pax7*−/−) of ctx. Starting from the day of injury, animals were subcutaneously injected with G-CSF (250 ng per 1 g of body weight).[27] G-CSF was administrated twice a day, every 12 h, for 4 days. Twenty four hours post injury muscles were injected with 10 ng/10 μL (*wt*) or 5 ng/5 μL (*Pax7*−/−) of Sdf-1. Contralateral muscles were injected with the same volume of saline. Next, 4 or 7 days after injury, mice were sacrificed and muscles were isolated. Additionally, peripheral blood mice was drawn from the animals at day 4 following injury. *Figure* 1A presents the experimental scheme.

Analysis of cell numbers associated with individual fibres

Four days after injury, muscle fibres were isolated from *wt* and *Pax7*−/− mice gastrocnemius muscles, according to the Rosenblatt method.[28] Briefly, muscles were digested with collagenase I for 1.5 h at 37°C. Next, single fibres were transferred to single wells of 96-well plates coated with 10% Matrigel characterized by reduced growth factors concentration (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA) and cultured in DMEM (Life Technologies) supplemented with 10% horse serum (HS, Life Technologies), 20% fetal bovine serum (FBS, Life Technologies), 0.5% chicken embryo extract (CEE, Sera Laboratories), and 1% antibiotic (AB, Life Technologies). After 48 h of culture, the numbers of cells that migrated off the individual fibres were counted. After 72 h of culture, the morphology of the cells was analyzed. Each culture was repeated five times.

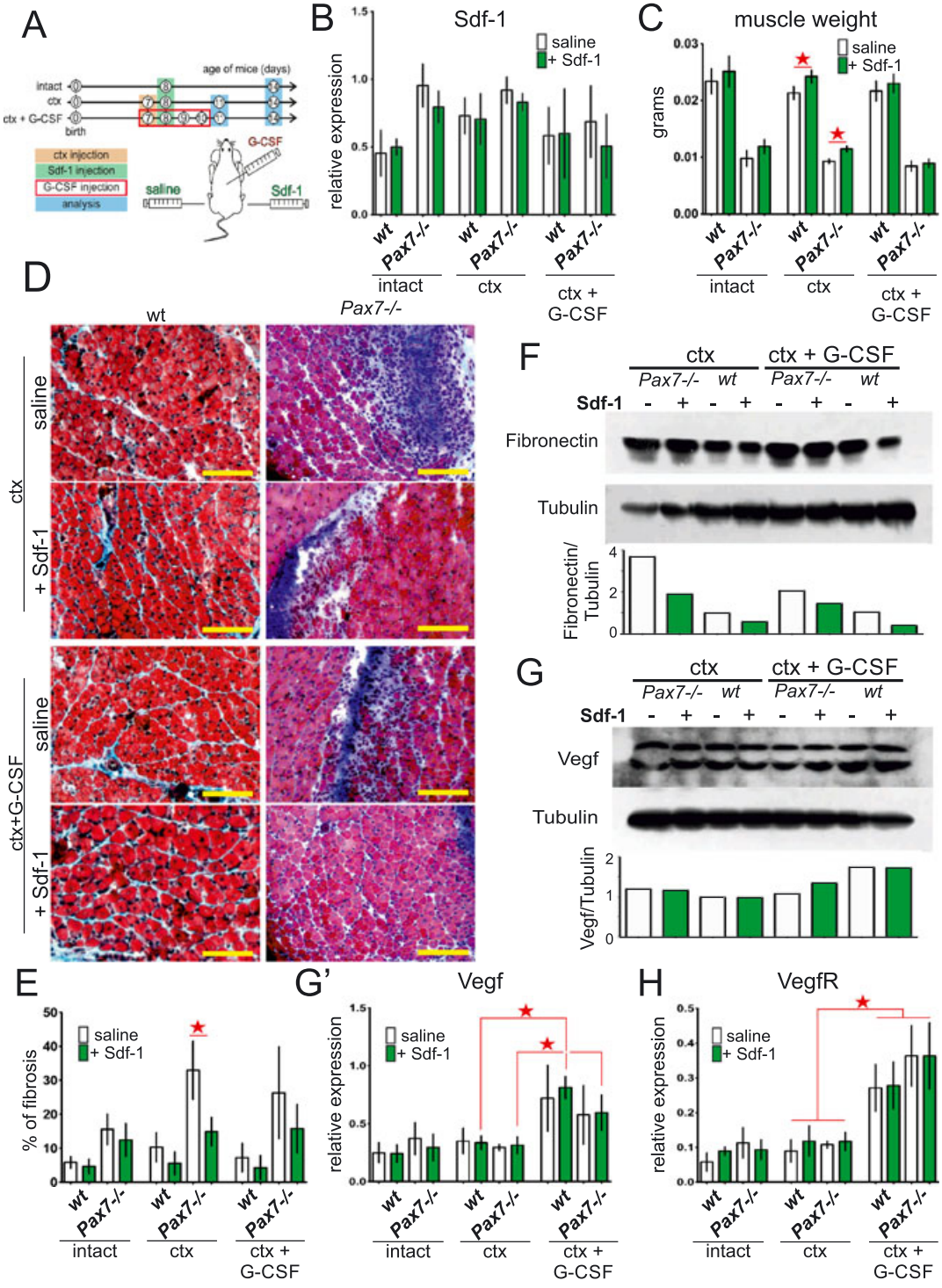
Bone marrow-derived stem cells migration assay

Whole population of bone marrow cells was obtained from *wt* and *Pax7*−/− mice. Bone marrow cells were washed in phosphate buffered saline (PBS) and centrifuged in Histopaque 1077 gradient (Sigma-Aldrich) to remove erythrocytes. Obtained mononucleated cells were seeded on culture inserts (8 μm pore size) coated with Matrigel containing reduced growth factor concentration (Becton Dickinson Bioscience) and cultured in α-MEM (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Life Technologies), 20% FBS, and 1% antibiotic (penicillin and streptomycin, Life Technologies). After 48 h of culture, non-adherent cells were removed. Next, culture medium present in the lower chamber was supplemented with Sdf-1 (50 ng/mL). After an additional 48 h of culture, the cells were fixed with methanol and stained with Giemsa (Merck, Darmstadt, Germany), according to the manufacturer's protocol. The number of cells that migrated through the insert pores and localized either at the insert surface facing the lower dish or at the bottom of the lower chamber was counted. Analysis was performed in triplicates.

Quantitation of mRNA levels

Total RNA was extracted from intact and injured muscles of *wt* and *Pax7*−/− mice using the RNasy Midi Kit (QIAGEN, Venlo, Netherlands), according to the manufacturer instruction. 500 ng of total RNA was reverse transcribed using the Transcriptor First Stand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany), according to the manufacturer's protocol. mRNA levels were examined using custom PCR array based on Universal ProbeLibrary (Roche Applied Science) for the following genes: *Cxcr4*, *Cxcr7*, *Sdf*-1, *CD34*, *CD45*, *CD133*, *Myf5*, *m-cadherin*, muscle creatine kinase (*m-CK*), vascular endothelial growth factor (*VEGF*), VEGF receptor (*VEGFR*), and embryonic myosin heavy chains (*eMyHC*). Hypoxanthine phosphoribosyltransferase 1 (*Hprt 1*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), and beta-2-microglobulin (*B2m*) were used as the candidate reference genes. Quantitative real-time PCR analysis was performed using the LightCycler 480 Probes Master 9.0 (Roche Applied Sciences) and LightCycler 480 (Roche Applied

Figure 1 Regeneration of intact and injured *wt* and *Pax7−/−* gastrocnemius muscles treated with stromal derived factor-1 (Sdf-1) or granulocyte-colony stimulating factor (G-CSF) and Sdf-1. Analyses were performed at day 7 of regeneration. (A) Experimental design. (B) The level of Sdf-1 mRNA normalized to the level of housekeeping genes. Data are the means ± SD. No statistically important differences were observed. (C) The muscle weight. The statistically important differences are marked with stars. (D) The muscle cross sections stained with Masson's trichrome. Bar 100 μm. (E) The level of fibrosis showed as proportion of tissue area occupied by connective tissue. The statistically important differences are marked with stars. (F) The level of fibronectin and tubulin protein. (G) The level of vascular endothelial growth factor (Vegf) and tubulin protein. (G') The level of Vegf mRNA normalized to the level of housekeeping genes. (H) The level of vascular endothelial growth factor receptor (VegfR) mRNA normalized to the level of housekeeping genes. The statistically important differences are marked with stars.



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Sciences), according to the PCR array manufacturer protocol. Threshold-cycle (Ct) values of the analyzed amplicons were determined using the LightCycler 480 Software (Roche Applied Science). Expression levels were calculated with the 2-(ΔCT) formula using the relative quantification tool in the LightCycler 480 Software. All the candidate reference genes displayed high expression stability and were therefore used for the normalization of the expression data. RNAs isolated from three muscles of each group were analyzed.

Western blot analysis

The protein lysates from muscles of 14-day-old *wt* and *Pax7*−/− mice were obtained using Complete Lysis-M EDTA-free reagent (Roche Applied Science) and denatured by boiling in Laemmli buffer. Next, 25 μg of protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Roche Applied Science), according to previously described protocol.[15] Membranes were washed in Tris-buffered saline (TBS), incubated in 5% non-fatty dry milk in TBS at room temperature for 1 h, followed by the overnight incubation in primary antibody diluted in 5% non-fatty dried milk in TBST (1:2 000) at 4°C. Next, the membranes were exposed for 2 h to HRP-conjugated secondary antibody diluted in 5% non-fatty dry milk in TBST (1:10 000 or 1:20 000) at room temperature. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to chemiluminescence positive film (Amersham Hyperfilm ECL, GE Healthcare, Little Chalfont, UK). The experiment was performed in triplicates, that is, three muscles of each group were independently analyzed. The protein level was quantified using the Gel Doc XR+ (BioRad, Hercules, CA, USA) and Image Software System version 5.1 (BioRad). The optical density of analyzed protein bands was compared with tubulin bands. The following primary antibodies were used: rabbit polyclonal anti-Cxcr4 (Abcam, Cambridge, UK), rat monoclonal (MEC 14.7) anti-CD34 (Abcam), rabbit polyclonal anti-Cxcr7 (Abcam), rabbit polyclonal anti-m-CK (Sigma-Aldrich), mouse monoclonal (EP5) anti-fibronectin (Santa Cruz Biotechnology, Dallas, TX, USA), and mouse monoclonal (B-5-1-2) anti-tubulin (Sigma-Aldrich), rabbit polyclonal anti-VEGF (Millipore, Billerica, MA, USA), and goat polyclonal anti-VEGFR (Santa Cruz Biotechnology). The following secondary antibodies were used: goat anti-mouse Igg (Sigma-Aldrich, dilution 1:20 000), goat anti-rabbit (Sigma-Aldrich, dilution 1:10 000), and goat anti-rat (Calbiochem, dilution 1:10 000).

Histochemical staining

Six days after Sdf-1 injection to intact and injured gastrocnemius muscles of *wt* and *Pax7*−/− mice, the muscles were dissected. Next, muscles were frozen in isopentane cooled with liquid nitrogen, transferred into −80°C, and cut into 7 μm-thick sections using cryomicrotome (Microm HM505N). Sections were hydrated in deionized water and fixed in Bouin's reagent (Sigma-Aldrich) overnight. Masson's Trichrome (Sigma-Aldrich) staining was performed according to the manufacturer protocol. Finally, sections were dehydrated and mounted with Entellan (Merck). Sections were photographed using Nikon Eclipse, TE200 microscope equipped with Hoffman contrast. Fields of view (10 per each variant) were analyzed using GIMP ImageJ to evaluate the percentage of the tissue area occupied by connective tissue. Five muscles collected during independent experiments were analyzed.

Immunolocalization

Gastrocnemius muscles of *wt* and *Pax7*−/− mice were frozen in isopentane cooled with liquid nitrogen, transferred into −80°C, cut into 7 μm-thick sections using cryomicrotome (Microm HM505N). Muscle sections were hydrated in PBS and fixed in 3% PFA for 10 min. Next, samples were washed with PBS, permeabilized with 0.05% TritonX100 (Sigma-Aldrich) in PBS, incubated in 0.25% glycine (Sigma-Aldrich) in PBS, followed by 1 h incubation in 3% BSA in PBS (Sigma-Aldrich) containing 2% donkey serum (Sigma-Aldrich) at room temperature. Next, all the samples were incubated at 4°C in primary antibodies diluted in 3% bovine serum albumin (BSA) in PBS (1:100), overnight. Subsequently, the specimen were incubated for 2 h with secondary antibodies conjugated with Alexa Fluor 488, 568, or 594 (Life Technologies) diluted in 1.5% BSA/PBS (1:200), at room temperature. In order to visualize the nuclei, samples were incubated for 5 min at room temperature with DraQ5 (Biostatus Limited, Leicestershire, GB) diluted in PBS (1:1000). Finally, the samples were mounted with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark).

Single muscle fibres, isolated according to the Rosenblatt method, were fixed for 10 min with 3% PFA in PBS. Then fibres were permeabilized in 0.5% TritonX100 (Sigma-Aldrich) for 10 min, incubated in 15 mM NH_4Cl for 20 min, followed by 30 min incubation with 10% HS (Life Technologies) at room temperature. Next, fibres were incubated overnight at 4°C with primary antibodies diluted in 10% HS in PBS (1:50), which was followed by 1 h of incubation with secondary antibodies conjugated with Alexa Fluor 488 or 594 (Life Technologies) diluted in 10% HS/PBS (1:100), at room temperature. Nuclei were visualized with DraQ5 (Biostatus Limited) diluted in PBS (1:1000), incubated at room temperature, for 5 min. Fibres were mounted with Fluorescent Mounting Medium (DakoCytomation).

The following primary antibodies were used: mouse monoclonal anti-CD45 (Santa Cruz Biotechnology), rabbit polyclonal anti-Cxcr4 (Abcam), rat monoclonal (MEC 14.7) anti-CD34 (Abcam), rabbit polyclonal anti-Cxcr7 (Abcam), rabbit

monoclonal (SP6) anti-Ki67 (Abcam), mouse monoclonal (12G4) anti-m-cadherin (Abcam), mouse monoclonal (F1.652) anti-eMyHC (Hybridoma Bank), and rabbit polyclonal anti-laminin (Sigma-Aldrich). The following secondary antibodies were used: donkey anti-mouse Igg Alexa 594 conjugated (Life Technologies), donkey anti-rabbit Igg Alexa 488 conjugated (Life Technologies), donkey anti-rabbit Igg Alexa 594 conjugated (Life Technologies), and goat anti-rat Igg Alexa 568 conjugated (Life Technologies). Appropriate controls of secondary antibodies were performed. Each analysis was repeated three times.

Flow cytometry analysis

Four days after the injury of gastrocnemius muscles of *wt* and *Pax7*−/− mice, the peripheral blood (250 μL) was collected into the heparin containing tubes. The erythrocytes were lysed using lysis buffer (0,17 M Tris HCl, 0,16 M NH_4Cl, pH 7.2). Simultaneously, gastrocnemius muscles were dissected and incubated for 2 h at 37°C in 0.2% collagenase I (Sigma-Aldrich), followed by 40 min incubation in 0.05% Dispase (Becton Dickinson Bioscience) at 37°C. Next, muscles were fragmented by pipetting using 20G needle. Obtained suspensions of cells and fibre fragments were filtered through a 40 μm cell strainer (Becton Dickinson Bioscience). Collected cells were incubated for 30 min with 0.5% BSA supplemented with 1% FBS in PBS, at room temperature. Next, cells were transferred to anti-CD34-PE (Abcam) or anti-Cxcr4 (Abcam) primary antibodies diluted 1:100 in PBS with 0.5% BSA and incubated for 30 min at 4°C, washed in PBS, and then incubated for 30 min with secondary Alexa 488 conjugated antibody diluted 1:200 (Life Technologies) at room temperature. Finally, both blood and muscle-derived cells were fixed for 10 min in 2% PFA in PBS, at room temperature. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson Bioscience). Control samples were incubated with isotype fluorochrome matched immunoglobulin antibodies. Each analysis was repeated five times.

Statistical analysis

Data was presented as means ± standard deviation. Student's *t*-test was used for statistical comparisons. $P < 0.05$ was considered significant and marked with an asterisk.

Results

Stromal derived factor-1 improves Pax7−/− muscles regeneration

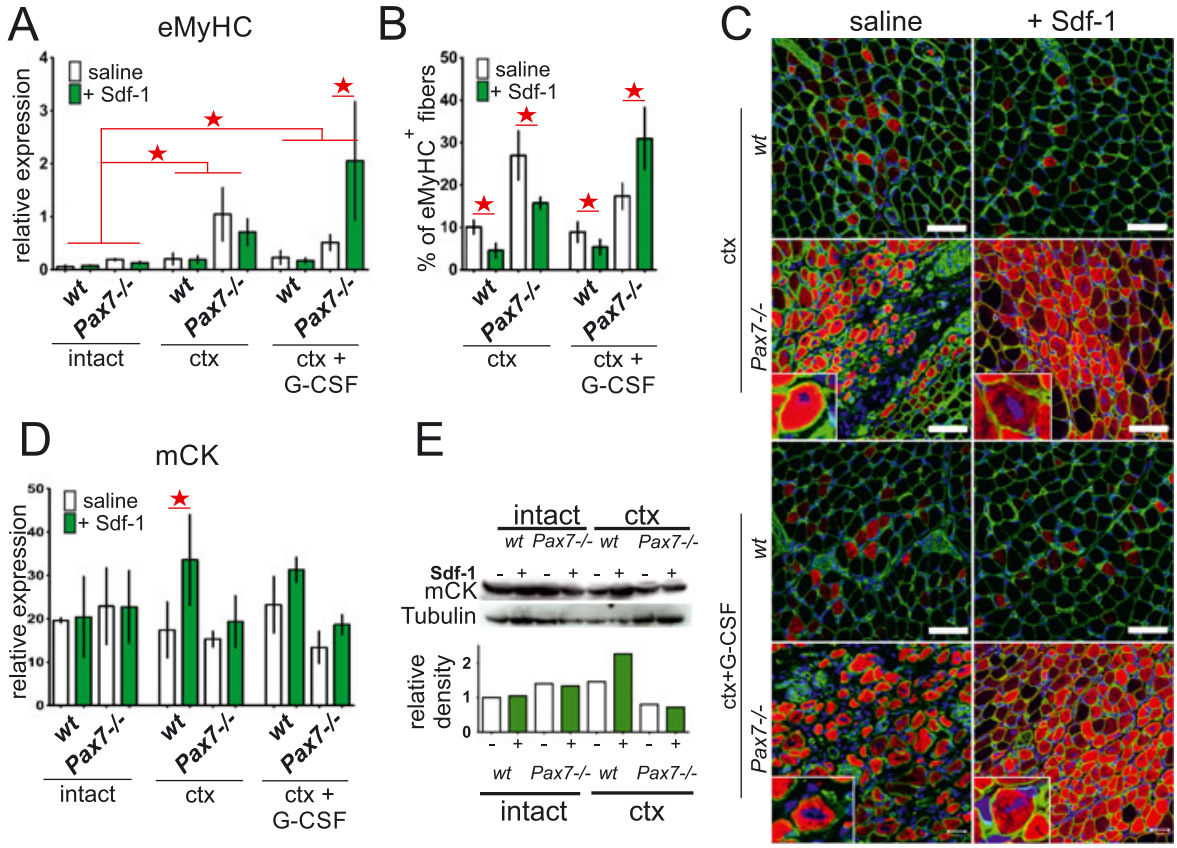
Our previous studies documented that Sdf-1 is produced during skeletal muscles regeneration[29], and exogenous Sdf-1 can improve this process.[15] Because this cytokine can increase the mobilization of stem cells into injured muscle, we decided to check whether it can also improve growth and regeneration of skeletal muscles of *Pax7*−/− mice, that is, mice lacking satellite cells. Thus, by using *Pax7*−/− mice, we analyzed whether the endogenous stem cells would be mobilized by Sdf-1 and would be able to substitute missing satellite cells. We also mobilized stem cells from bone marrow to peripheral blood using G-CSF. It allowed us to increase the number of stem cells possibly able to engraft the injured muscles. To this point, intact or ctx damaged gastrocnemius muscles of *wt* and *Pax7*−/− mice were injected either with saline (control) or Sdf-1. Ctx injured muscles were also treated with G-CSF (*Figure* 1A). To sumarize, the muscles of *wt* and *Pax7*−/− mice were analyzed in the following experimental variants: (1) intact control and Sdf-1 injected (intact); (2) ctx injured control and Sdf-1 injected (ctx); and (3) ctx injured and G-CSF treated control and Sdf-1 injected (ctx + G-CSF) (*Figure* 1A). Three and 6 days after Sdf-1 administration, that is, in either 11 or 14-day-old mice, we compared the progress and the 'quality' of growth as well as muscle regeneration.

The levels of Sdf-1 transcripts (endogenous Sdf-1) did not differ between the muscles of 14-day-old mice, that is, at day 7 of regeneration after ctx injection (*Figure* 1B). Importantly, the *Pax7*−/− muscle mass and muscle fibres diameter were smaller when compared with the *wt* muscles. All *wt* and *Pax7*−/− muscles, that is, intact and regenerating that were treated with Sdf-1, had a tendency to increase their mass when compared with the control, that is, saline injected ones (*Figure* 1C). Statistically significant differences ($P < 0.05$) were observed only in the case of *wt* and *Pax7*−/− mice that were injured and treated with only Sdf-1 (*Figure* 1C). G-CSF treatment did not have an effect on muscle mass. However, treatment with Sdf-1 and G-CSF, alone or in combination, significantly increased the ability of *Pax7*−/− muscle to reconstruct its structure, as shown in muscle cross sections stained with Masson's trichrome (*Figure* 1D). The regeneration of untreated injured *Pax7*−/− muscles (ctx and saline; *Figure* 1D) was defective, clearly indicating regeneration deficit at this stage (14-day-old mice, day 7 of regeneration after ctx injection). At the same time, regeneration of injured *Pax7*−/− muscles treated with Sdf-1 proceeded more efficiently (*Figure* 1D). Visualization of connective tissue in *wt* and *Pax7*−/− muscles with Masson's trichrome showed limited fibrosis in Sdf-1 treated muscles (*Figure* 1D). Importantly, the connective tissue area had a tendency to decrease in all injured muscles injected with Sdf-1, regardless of mice genotype (*Figure* 1E). Statistically significant differences ($P < 0.05$) in the fibrosis level were observed between injured *Pax7*−/− muscles and treated with Sdf-1 muscles. In addition, the level of fibronectin, which is characteristic for connective tissue, was lower in Sdf-1 injected muscles (*Figure* 1F). In order to verify whether Sdf-1 and G-CSF treatment enhances angiogenesis, we assessed the angiogenic factor levels, that is, the

transcript levels of Vegf and its receptor VegfR. The levels of Vegf and VegfR did not differ significantly between saline and Sdf-1 treated muscles of 14-day-old mice, that is, at day 7 of regeneration (*Figure* 1G, 1G′, and 1H and *Figure* S1A in the Supporting Information). Thus, it suggests that Sdf-1 did not influence the blood vessel formation in regenerating muscles. Importantly, G-CSF stimulation significantly increased the level of Vegf and VegfR, implying that G-CSF stimulation improves angiogenesis (*Figure* 1G, 1G′, 1H and *Figure* S1A).

Many lines of evidence showed that eMyHC is synthesized in newly formed muscle fibres.[30,31] For this reason, the augmentation of eMyHC expression indicates an increase of newly formed fibres number within the muscle. In intact *wt* and *Pax7*−/− muscles, the level of mRNA encoding eMyHC was very low (*Figure* 2A). At the seventh day of regeneration, ctx injury caused the increase in *eMyHC* transcript level in *wt* and *Pax7*−/− muscles (*Figure* 2A). Sdf-1 treatment insignificantly decreased eMyHC transcript levels in *Pax7*−/− muscles. Interestingly, G-CSF stimulation resulted in dramatic increase of *eMyHC* mRNA level in Sdf-1 treated *Pax7*−/− ($P < 0.05$) but not *wt* muscles (*Figure* 2A). Immunolocalization of eMyHC protein in regenerating *wt* and *Pax7*−/− muscles confirmed results of qPCR analysis at the protein level. The number of muscle fibres expressing eMyHC is presented in *Figure* 2B and C as their percentage in a total number of muscle fibres in the field of view (six muscles, 10 fields of view per muscle). Analysis of ctx injured *wt* muscles revealed low number of eMyHC positive fibres that decreased as a consequence of Sdf-1 treatment. *Pax7*−/− muscles were characterized by significantly higher number of eMyHC positive fibres, which also decreased following Sdf-1 treatment (*Figure* 2B and C). Surprisingly, G-CSF and Sdf-1 injections led to the dramatic increase in the formation of new, that is, immature, muscle fibres in *Pax7*−/− but not in *wt* muscles (*Figure* 2B and 2C). Moreover, the level of m-CK, which expression is associated with muscle fibres maturation, was

Figure 2 Expression and localization of muscle specific factors in intact and injured *wt* and *Pax7*−/− gastrocnemius muscles treated with stromal derived factor-1 (Sdf-1) or granulocyte-colony stimulating factor (G-CSF) and Sdf-1. Analyses were performed at day 7 of regeneration. (A) The level of embryonic myosin heavy chains (eMyHC) mRNA normalized to the level of housekeeping genes. Data are the means ± SD. The statistically important differences marked with stars. (B) The proportion of muscle fibres number expressing eMyHC protein (eMyHC$^+$ fibres) of all muscle fibres at cross section. The statistically important differences marked with stars. (C) Immunolocalization of eMyHC in newly formed muscle fibres. Green—laminin, red—eMyHC, and blue—nuclei. Bar 50 μm. (D) The level of muscle creatine kinase (mCK) mRNA normalized to the level of housekeeping genes. Data are the means ± SD. The statistically important differences marked with stars. (E) The level and analysis of mCK and tubulin protein.



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higher in ctx injured and Sdf-1 treated *wt* muscles compared with untreated muscles (*Figure* 2D and 2E). Taken together, this data indicates that Sdf-1 accelerates skeletal muscle regeneration in *wt* and *Pax7*−/− mice. G-CSF stimulation followed by Sdf-1 treatment dramatically changed the process of *Pax7*−/− muscle regeneration and led to massive formation of new immature muscle fibres (*Figure* 2B and 2C). Importantly, maturation of these newly formed muscle fibres was delayed in *Pax7*−/− mice.

Granulocyte-colony stimulating factor and stromal derived factor-1 treatment increases the mobilization of the stem cells into injured muscles

Next, we attempted to identify endogenous stem cells that would replace lacking satellite cells and improve skeletal muscle regeneration in *Pax7*−/− mice. Histological analysis revealed that Sdf-1 injection resulted in the increase of mononucleated cells localized between muscle fibres (*Figure* 1D), as compared with control ones (*Figure* 3A). G-CSF treatment further increased the number of mononucleated cells that engrafted ctx injured muscles, as compared with those treated only with Sdf-1 (*Figure* 3A). Next, the expression of mRNAs encoding stem cells markers, that is, CD34, Cxcr4, and Cxcr7, was significantly increased in G-CSF and Sdf-1 injected regenerating muscles, as compared with saline treated ones (*Figure* 3B). The level of mRNA encoding myogenic cells marker, that is, *Myf5*, was very low in *Pax7*−/− as compared with *wt* muscles. In intact and ctx injured muscles, neither Sdf-1 alone nor in combination with G-CSF increased Myf5 expression, regardless of mice genotype (*Figure* 3B). Analysis of mRNA encoding m-cadherin, another satellite cells marker, revealed that its level was increased only in Sdf-1-treated regenerating *wt* muscles (*Figure* 3B). In *Pax7*−/− muscles, m-cadherin expression did not change, proving that their regeneration depends on cells other than satellite cells or myoblasts derived from them (*Figure* 3B). Histological analysis revealed that the number of CD34 and Cxcr4 expressing cells and levels of these proteins in tissue lysates were higher in both *wt* and *Pax7*−/− muscles treated with Sdf-1, as well as co-stimulated with G-CSF and Sdf-1 as compared with untreated muscles (*Figure* 4A and 4B). The effect of G-CSF and Sdf-1 treatment on Cxcr7 expression in muscles was less pronounced (*Figure* 4B).

Figure 3 Analysis of mononucleated cells present in intact and injured *wt* and *Pax7*−/− gastrocnemius muscles treated with stromal derived factor-1 (Sdf-1) or granulocyte-colony stimulating factor (G-CSF) and Sdf-1. Analyses were performed at day 7 of regeneration. (A) The number of mononucleated cell counted on muscle cross sections stained with Masson's trichrome. The statistically important differences marked with stars. (B) The level of CD34, Cxcr4, Cxcr7, Myf5 and m-cadherin mRNA normalized to the level of housekeeping genes. The statistically important differences marked with stars.

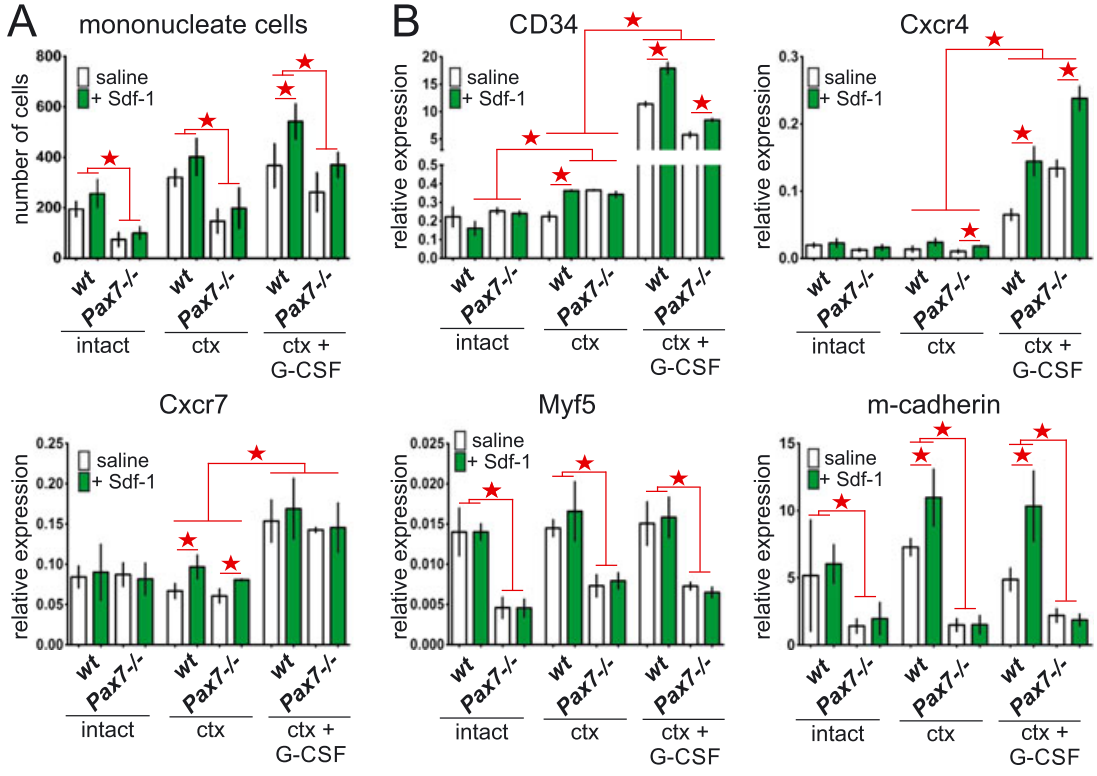

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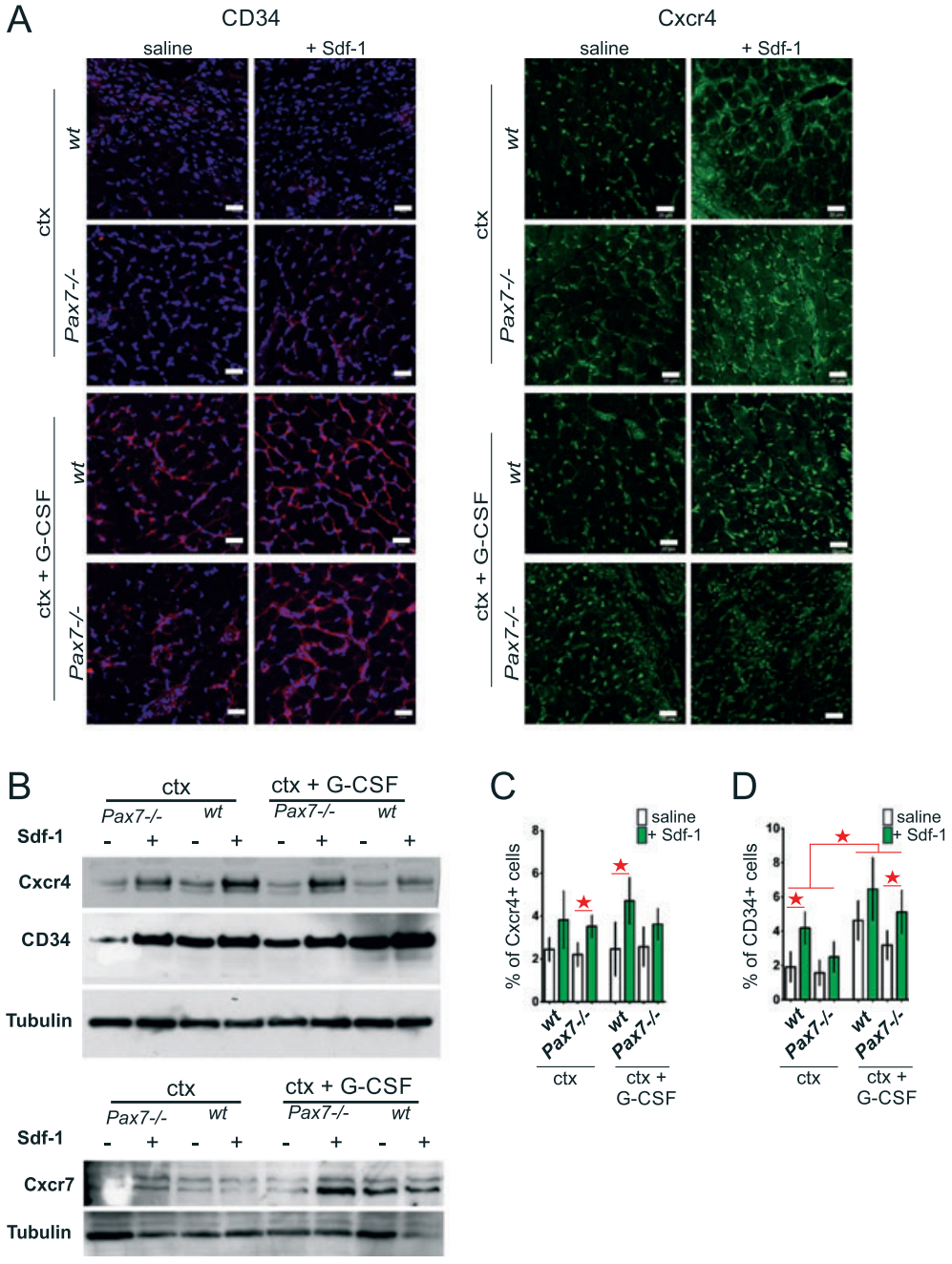
Figure 4 Analysis of localization and level of CD34 and Cxcr4 in cells present in intact and injured *wt* and *Pax7*−/− gastrocnemius muscles treated with stromal derived factor-1 (Sdf-1) or granulocyte-colony stimulating factor (G-CSF) and Sdf-1**.** A. The presence of CD34 and Cxcr4 positive cells in muscles at day 7 of regeneration. Green—Cxcr4, red—CD34, and blue—nuclei. Bar 20 μm. (B) The level of Cxcr4, CD34, Cxcr7, and tubulin protein at day 7 of regeneration. (C) The proportion of Cxcr4 positive cells of all cells isolated from regenerating muscle measured by fluorescence-activated cell sorting at day 4 of regeneration. (D) The proportion of CD34 positive cells of all cells isolated from regenerating muscle measured by FACS at day 4 of regeneration.



To exclude the possibility that Sdf-1 increased the expression of Cxcr4 and CD34 in cells already present within the muscle, we decided to isolate mononucleated cells from *wt* and *Pax7*−/− muscles at day 4 after injury and determine the proportion of Cxcr4 or CD34 expressing cells by fluorescence-activated cell sorting (FACS) analysis (*Figure* 4C and 4D). In all samples obtained from either Sdf-1 or G-CSF and Sdf-1 treated muscles, we observed the tendency that the number of Cxcr4 and CD34 positive cells was higher, as compared with non-treated muscles. To exclude the

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possibility that Sdf-1 and G-CSF stimulate cell proliferation but not their migration, we assessed the number of proliferating cells, that is, expressing Ki67, in regenerating muscles. The number of Ki67 expressing cells was found to be comparable in all the analyzed muscles at day 7 of regeneration (*Figure* 5A). Thus, neither G-CSF nor Sdf-1 affected the proliferation rate of resident cells but increased the number of cells by promoting the homing of circulating cells into the regenerating muscles. This result was confirmed by the analysis of muscle fibres that were isolated from *wt* and *Pax7*−/− gastrocnemius muscles at day 7 of regeneration. Co-localization of Ki67 and m-cadherin in the cells that were attached to muscle fibres showed that the proportion of double immunostained ones, that is, expressing both Ki67 and m-cadherin, was comparable regardless of mice genotype and type of muscle treatment (*Figure* 5B). Thus, we confirmed that G-CSF and Sdf-1 did not affect the proliferation of resident cells, that is, satellite cells. In addition, we checked the presence of so-called inflammatory cells within the analyzed muscles. The level of mRNA encoding CD45, which is characteristic for inflammatory cells, did not change in response to G-CSF and Sdf-1 (*Figure* 5C), indicating that the cells present within the muscle were not inflammatory ones. Additionally, immunostaing for CD45 (*Figure* S1B) showed that CD45+ cells were rarely observed within the analyzed muscle. Thus, we suggest that at day 7 of regeneration, infiltration of muscle tissue by immune system cells is low. Moreover, the level of CD133 marker characteristic for circulating stem cells (AC133+) did not differ between control and treated muscles (*Figure* 5D).

The source of the stem cells mobilized by stromal derived factor-1 and granulocyte-colony stimulating factor into injured muscles

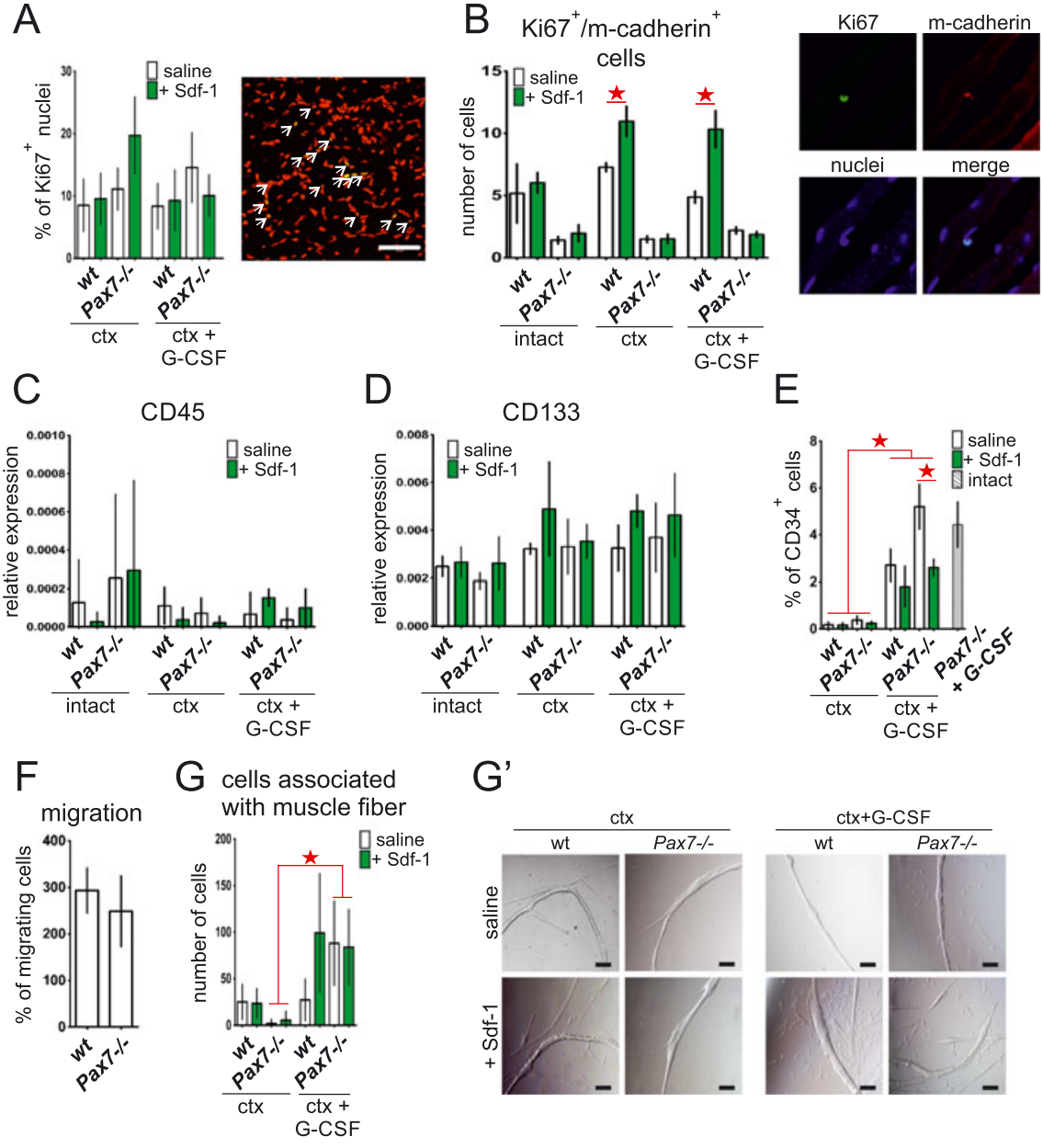
To verify whether in our hands G-CSF promotes the release of bone marrow stem cells (BMSCs) into circulation, we analyzed the peripheral blood for the presence of CD34 expressing cells. We did not detect any increase in CD34 positive cells in the blood of *wt* and *Pax7*−/− mice following muscle injury with ctx, regardless of Sdf-1 treatment. However, G-CSF was found to cause an increase in the number of CD34 expressing cells in the peripheral blood of *wt* and *Pax7*−/− mice, analyzed at day 4 of regeneration (*Figure* 5E). The number of these cells was lower in those mice in which ctx-treated muscles were Sdf-1 injected, suggesting their increased homing to the site of injury. We also observed that G-CSF caused an increase in the number of CD34 positive cells in blood of *Pax7*−/− mice which muscles were not injured (*Figure* 5E, grey bar). Next, we compared ability of BMSCs of *wt* and *Pax7*−/− mice to migrate in Sdf-1 gradient. Transmembrane migration assay showed that *wt* and *Pax7*−/− BMSCs cells did not differ in their migration potential (*Figure* 5F). Further analysis covered the isolation of muscle fibres and cells from *wt* and *Pax7* −/− muscles at day 4 of regeneration. These fibres were cultured individually for 48 h, and cells that migrated out of them were counted (*Figure* 5G and 5G'). The number of cells present in the vicinity of the analyzed fibres was higher in fibres of mice injected with G-CSF and Sdf-1, as compared with fibres isolated from Sdf-1 or saline injected mice, regardless of their genotype.

Discussion

Our previous studies provided the evidence that Sdf-1 stimulation improves skeletal muscle regeneration. This effect can be linked to the presence of Cxcr4 and CD34 expressing cells and also to the increase of myoblast migration capacity in metalloproteinase-2 (MMP-2) and MMP-9-dependent manner.[15] Available data, however, do not provide straightforward proof showing that regeneration process can depend on endogenous cells other than satellite cells. To verify such notion, we studied mice carrying a targeted mutation in the *Pax7* gene (*Pax7*−/−). Pax7 has been shown to inhibit satellite cells differentiation by inhibiting Myod1-dependent activation of myogenin that is a crucial myogenic transcription factor.[32] Another myogenic transcription factor, that is, *Myf5*, is also a direct target gene of Pax7.[33] Previous study showed that *Pax7*−/− mice are significantly smaller than *wt* and are characterized by a limited lifetime.[34] We also showed here that *Pax7*−/− muscles, such as gastrocniemus, had significantly lower mass comparing with *wt* ones. This phenotype is caused by the reduced number of satellite cells present in the skeletal muscles of *Pax7*−/− mice. Satellite cells from *Pax7*−/− mice are characterized by cell cycle arrest, inability to proliferate, and precocious differentiation.[23,24,35,36] Loss of satellite cells in *Pax7*−/− mice is a progressive process starting soon after the animal birth. In 10-day-old *Pax7*−/− mice, satellite cell number drops to 15–25% of the number present in newborn mice.[35,36] As a result, the satellite cells are hardly detectable. In adult *Pax7*−/− mice, that is, 8 weeks old.[24] *Pax7*−/− mice are also characterized by reduced muscle fibre size. This suggests defective phase of postnatal skeletal muscle growth in these animals, while it is known that this phase in *wt* mice is mediated by satellite cells.[23,35,36]

As shown by Lepper and coworkers, the role of Pax7 could be dependent on mouse age. Ablation of Pax7 expressing cells during postnatal growth (between 7 and 18 days of age) leads to the complete inhibition of regeneration. However, in adult mice (older than 21 days), Pax7 positive cells appear not to be essential for muscle regeneration, and their ablation does not inhibit regeneration.[37] On the other hand, Rudnicki's group showed that inactivation of Pax7 in satellite cells of adult mice, that is, 40 days old, markedly impaired muscle regeneration.[38] Other authors report that in *Pax7*−/− mice older than 21 days, the regeneration process is reduced[24,36] proving the substantial role of satellite cells in skeletal muscle regeneration. This function of satellite cells

Figure 5 Analysis of Ki67 positive cells (Ki67$^+$) in intact and injured *wt* and *Pax7−/−* gastrocnemius muscles treated with stromal derived factor-1 (Sdf-1) or granulocyte-colony stimulating factor (G-CSF) and Sdf-1 (A and B). (E) Mobilization and migration ability of cells isolated at day 4 and 7 of regeneration (C–G'). (A) The proportion of Ki67 positive nuclei (graph) of all nuclei at cross section and representative immunolocalization of Ki67 in *wt* muscle at day 7 of regeneration. Red—nuclei, green—Ki76, and yellow—colocalization. Bar 50 μm. The statistically important differences marked with stars. (B) The number of Ki67 and m-cadherin positive cells connected with freshly isolated muscle fibres (graph) and immunolocalization of Ki67 and m-cadherin at day 7 of regeneration. Bar 10 μm. The statistically important differences marked with stars. (C) The level of CD45 mRNA at day 7 of regeneration, normalized to the level of housekeeping genes. The statistically important differences marked with stars. (D) The level of CD133 mRNA at day 7 of regeneration, normalized to the level of housekeeping genes. The statistically important differences marked with stars. (E) The proportion of CD34 positive cells (CD34$^+$) in peripheral blood at day 4 measured by fluorescence-activated cell sorting. The statistically important differences marked with stars. (F) The migration of *wt* and *Pax7−/−* BMSCs in Sdf-1 gradient (transmembrane migration assay). The statistically important differences marked with stars. (G) The number of cells associated with isolated at day 4 of regeneration muscle fibres after 48 h of *in vitro* culture. The statistically important differences marked with stars. (G') The presence of cells associated with muscle fibre after 48 h of *in vitro* culture. Transmitted light images. Bar 100 μm.



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was postulated from the very moment of their discovery.[39] The essential role of satellite cells in adult muscle regeneration was also demonstrated in other studies.[40,41]

Our work proves that defects in Pax7−/− muscle regeneration can be ameliorated. By analyzing regenerating muscles of 14-day-old *Pax7*−/− mice, we showed that their regeneration is impaired and associated with the development of fibrosis. However, by using G-CSF and then Sdf-1, we were able to induce the mobilization of stem cells to the injured muscles, ameliorated fibrosis and, in a consequence, made the skeletal muscle regeneration effective, even in the absence of satellite cells. The improvement of regeneration was manifested in better muscle architecture, higher creatine kinase levels, and lower fibrosis. Moreover, we observed that G-CSF and Sdf-1 treatment led to the increase of VegfR level that could suggest amelioration of angiogenesis. Indeed, it was previously shown that combination of G-CSF and Sdf-1 treatments enhanced neovascularization in ischemic muscles.[42] This angiogenic effect can be another factor, apart from mobilization of stem cells, improving reconstruction of regenerating muscles.

In growing *Pax7*−/− skeletal muscles, the number of satellite cells progressively decreases. Thus, in case of muscle injury, improved muscular regeneration has to result from stem cells mobilization. This hypothesis was proved by detailed analyses of the cells present within regenerating muscles of mice stimulated with G-CSF and Sdf-1. A very limited number of previous studies documented that endogenous (not transplanted) stem cells could, albeit with low frequency, participate in the skeletal muscle reconstruction. First, it was shown that muscle resident pericytes were able to contribute to regeneration and also colonize the satellite cell niche.[43] Next, it was demonstrated that endogenous bone marrow cells with very low frequency can took part in the muscle regeneration.[44] Our results suggest that *Pax7*−/− BMSCs are able to react to Sdf-1 treatment and improve muscle regeneration showing that they can participate in this process regardless of functional Pax7. Furthermore, another study suggested that BMSCs are able to undergo myogenic differentiation in *Pax7* independent manner.[45]

The main population of cells mobilized to injured *wt* and *Pax7*−/− skeletal muscles were CD34 and Cxcr4 positive ones (but CD133 and CD45 negative). Importantly, as it was shown by Ieronimakis and coworkers, the proportion of CD34 expressing cells remains constant after mouse muscle injury.[46] Apart from the endothelial progenitors and stromal cells, CD34 expression characterizes haematopoietic stem and progenitor cells (reviewed in Lin *et al.*[47]). A low number of CD34 positive cells is present in the peripheral blood and could be significantly increased by a mobilization protocol based on G-CSF administration (reviewed in Moggatt and Pelus[48]). The reports concerning the influence of G-CSF on myoblasts proliferation are conflicting. Some data document that G-CSF does not influence myoblasts proliferation or differentiation *in vitro*.[49] Other lines of evidence imply that G-CSF receptor is expressed in C2C12 myoblasts, and that G-CSF strongly induces myoblasts proliferation *in vitro*.[50] Moreover, the expression of G-CSF receptor was shown to be elevated in regenerating muscle in mice, starting from day 3 to 8 after ctx injury, that is, at the stages when myoblasts proliferate.[50] Importantly, mice lacking functional G-CSF receptor gene were characterized by diminished regeneration, and G-CSF administration led to an increase of the number of myoblasts and improved skeletal muscle regeneration.[50] Results of our experiments indicate, however, that G-CSF used together with Sdf-1 does not affect the proliferation of cells mobilized into the regenerating muscles of *Pax7*−/− mice at analyzed stage of regeneration. In *wt* mice, such treatment increased the number of cells expressing Ki67 together with m-cadherin, suggesting that it stimulates only satellite cells-derived myoblasts. We did not dissociate, however, the action of G-CSF from Sdf-1.

As we have demonstrated, the administration of G-SCF and Sdf-1 immediately after muscle injury can be very effective, even in the case of skeletal muscles lacking satellite cells. Fibrosis was diminished, and endogenous cells were mobilized to the regenerating tissue. This observation can be remove used during the development of the treatment of many muscle diseases and impairments. However, as it was shown previously, the frequency of the formation of new fibres with the participation of transplanted bone marrow stem cells is very low [e.g. Corbel *et al.*[51]], suggesting that the major population of mobilized cells is not highly effective in supporting skeletal muscle regeneration. This can be a limiting factor for the therapies based on such cells. In the current study, we showed that single administration of Sdf-1 could be an effective stimulant increasing the participation of BMSCs in the skeletal muscle regeneration. Additional increase in the number of stem cells present in the blood stream, achieved by G-CSF injections, allows for the improvement of the regeneration process. To conclude, the muscle regeneration can be improved by treatment with Sdf-1 alone or combined with G-CSF, even when the population of skeletal muscle specific stem cells, that is, satellite cells is nearly exhausted. Our results add to the currently existing knowledge on the use of mouse model lacking satellite cells for studying novel therapies of pathological conditions of muscles with reduced satellite cell numbers.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher website:

Figure S1. A. Regeneration of intact and injured wt and *Pax7–/–* gastrocnemius muscles treated with Sdf-1 or G-CSF and Sdf-1. Analyses were performed at day 7 of regeneration and showed the level of VegfR and tubulin protein. B. Localization of CD45 positive cells in *wt* gastrocnemius muscles at day 7 of regeneration. Blue – cells nuclei, green – laminin, red – CD45.

Conflict of interest

K.K., A.R., A.K., S.W., P.A., G.M., C.M.A., and B.E. declare that they have no conflict of interest.

References

1. Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* 2012;**139**:2845–2856.
2. Rigamonti E, Zordan P, Sciorati C, Rovere-Querini P, Brunelli S. Macrophage plasticity in skeletal muscle repair. *Bio Med research international* 2014;**2014**:560629.
3. Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, *et al.* Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp Cell Res* 2002;**281**:39–49.
4. Moss FP, Leblond CP. Satellite cells as the source of nuclei in muscles of growing rats. *Anat Rec* 1971;**170**:421–435.
5. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, *et al.* Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* 2005;**122**:289–301.
6. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature* 2008;**456**:502–506.
7. Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature* 1989;**337**:176–179.
8. Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttmann R, Holland P, *et al.* Myoblast transfer in Duchenne muscular dystrophy. *Ann Neurol* 1993;**34**:8–17.
9. Briggs D, Morgan JE. Recent progress in satellite cell/myoblast engraftment—relevance vfor therapy. *FEBS J* 2013;**280**:4281–4293.
10. Skuk D, Goulet M, Roy B, Piette V, Cote CH, Chapdelaine P, *et al.* First test of a "high-density injection" protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up. *Neuromuscul Disord* 2007;**17**:38–46.
11. Galvez BG, Sampaolesi M, Brunelli S, Covarello D, Gavina M, Rossi B, *et al.* Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. *J Cell Biol* 2006;**174**:231–243.
12. Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, *et al.* Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. *Nat Commun* 2011;**2**:499.
13. Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, *et al.* Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. *J Clin Invest* 2004;**114**:182–195.
14. Cencioni C, Capogrossi MC, Napolitano M. The SDF-1/CXCR4 axis in stem cell preconditioning. *Cardiovasc Res* 2012;**94**:400–407.
15. Brzoska E, Kowalewska M, Markowska-Zagrajek A, Kowalski K, Archacka K, Zimowska M, *et al.* Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. *Biol Cell* 2012;**104**:722–737.
16. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 1997;**185**:111–120.
17. Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, *et al.* Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells* 2006;**24**:1254–1264.
18. Elmadbouh I, Haider H, Jiang S, Idris NM, Lu G, Ashraf M. Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. *J Mol Cell Cardiol* 2007;**42**:792–803.
19. Kuliszewski MA, Kobulnik J, Lindner JR, Stewart DJ, Leong-Poi H. Vascular gene transfer of SDF-1 promotes endothelial progenitor cell engraftment and enhances angiogenesis in ischemic muscle. *Mol Ther* 2011;**19**:895–902.
20. Alvarez P, Carrillo E, Velez C, Hita-Contreras F, Martinez-Amat A, Rodriguez-Serrano F, *et al.* Regulatory systems in bone marrow for hematopoietic stem/progenitor cells mobilization and homing. *BioMed research international* 2013;**2013**:312656, doi:10.1155/2013/312656.
21. Hoggatt J, Pelus LM. Mobilization of hematopoietic stem cells from the bone marrow niche to the blood compartment. *Stem cell research & therapy* 2011;**2**:13.
22. Fu ZH, Dong W, Gai LY, Wang F, Ding R, Chen YD. [Effect of erythropoietin combined with granulocyte-colony stimulating factor in the treatment of acute myocardial infarction in rats]. Nan fang yi ke da xue xue bao = *J Southern Med Univ* 2011;**31**:17–22.
23. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. *Cell* 2000;**102**:777–786.
24. Oustanina S, Hause G, Braun T. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. *Embo J* 2004;**23**:3430–3439.
25. Mansouri A, Stoykova A, Torres M, Gruss P. Dysgenesis of cephalic neural crest derivatives in Pax7−/− mutant mice. *Development* 1996;**122**:831–838.
26. Lu A, Cummins JH, Pollett JB, Cao B, Sun B, Rudnicki MA, *et al.* Isolation of myogenic progenitor populations from Pax7-deficient skeletal muscle based on adhesion characteristics. *Gene Ther* 2008;**15**:1116–1125.
27. Sicinska E, Lee YM, Gits J, Shigematsu H, Yu Q, Rebel VI, *et al.* Essential role for cyclin D3 in granulocyte colony-stimulating factor-driven expansion of neutrophil granulocytes. *Mol Cell Biol* 2006;**26**:8052–8060.
28. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. *In Vitro Cell Dev Biol Anim* 1995;**31**:773–779.
29. Brzoska E, Grabowska I, Hoser G, Streminska W, Wasilewska D, Machaj EK, *et al.* Participation of stem cells from human cord blood in skeletal muscle regeneration of SCID mice. *Exp Hematol* 2006;**34**:1262–1270.
30. Karsch-Mizrachi I, Travis M, Blau H, Leinwand LA. Expression and DNA sequence analysis of a human embryonic skeletal muscle myosin heavy chain gene. *Nucleic Acids Res* 1989;**17**:6167–6179.

31. Silberstein L, Webster SG, Travis M, Blau HM. Developmental progression of myosin gene expression in cultured muscle cells. *Cell* 1986;**46**:1075–1081.
32. Olguin HC, Yang Z, Tapscott SJ, Olwin BB. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *J Cell Biol* 2007;**177**:769–779.
33. Soleimani VD, Punch VG, Kawabe Y, Jones AE, Palidwor GA, Porter CJ, *et al.* Transcriptional dominance of Pax7 in adult myogenesis is due to high-affinity recognition of homeodomain motifs. *Dev Cell* 2012;**22**:1208–1220.
34. Mansouri A, Hallonet M, Gruss P. Pax genes and their roles in cell differentiation and development. *Curr Opin Cell Biol* 1996;**8**:851–857.
35. Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, *et al.* Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 2006;**172**:91–102.
36. Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *J Cell Biol* 2006;**172**:103–113.
37. Lepper C, Conway SJ, Fan CM. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature* 2009;**460**:627–631.
38. von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci U S A* 2013;**110**:16474–16479.
39. Mauro A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 1961;**9**:493–495.
40. Sambasivan R, Yao R, Kissenpfennig A, Van Wittenberghe L, Paldi A, Gayraud-Morel B, *et al.* Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development* 2011;**138**:3647–3656.
41. Lepper C, Partridge TA, Fan CM. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* 2011;**138**:3639–3646.
42. Tan Y, Shao H, Eton D, Yang Z, Alonso-Diaz L, Zhang H, *et al.* Stromal cell-derived factor-1 enhances pro-angiogenic effect of granulocyte-colony stimulating factor. *Cardiovasc Res* 2007;**73**:823–832.
43. Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, *et al.* Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol* 2007;**9**:255–267.
44. Palermo AT, Labarge MA, Doyonnas R, Pomerantz J, Blau HM. Bone marrow contribution to skeletal muscle: a physiological response to stress. *Dev Biol* 2005;**279**:336–344.
45. Xynos A, Corbella P, Belmonte N, Zini R, Manfredini R, Ferrari G. Bone marrow-derived hematopoietic cells undergo myogenic differentiation following a Pax-7 independent pathway. *Stem Cells* 2010;**28**:965–973.
46. Ieronimakis N, Balasundaram G, Rainey S, Srirangam K, Yablonka-Reuveni Z, Reyes M. Absence of CD34 on murine skeletal muscle satellite cells marks a reversible state of activation during acute injury. *PLoS One* 2010;**5**:e10920.
47. Lin CS, Ning H, Lin G, Lue TF. Is CD34 truly a negative marker for mesenchymal stromal cells? *Cytotherapy* 2012;**14**:1159–1163.
48. Hoggatt J, Pelus LM. Many mechanisms mediating mobilization: an alliterative review. *Curr Opin Hematol* 2011;**18**:231–238.
49. Wright CR, Brown EL, Della-Gatta PA, Ward AC, Lynch GS, Russell AP. G-CSF does not influence C2C12 myogenesis despite receptor expression in healthy and dystrophic skeletal muscle. *Frontiers in physiology* 2014;**5**:170.
50. Hara M, Yuasa S, Shimoji K, Onizuka T, Hayashiji N, Ohno Y, *et al.* G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation. *J Exp Med* 2011;**208**:715–727.
51. Corbel SY, Lee A, Yi L, Duenas J, Brazelton TR, Blau HM, *et al.* Contribution of hematopoietic stem cells to skeletal muscle. *Nat Med* 2003;**9**:1528–1532.

Fuzja komórek macierzystych z komórkami mięśniowymi w odpowiedzi na stymulację Sdf-1

Publikacja oryginalna 2: *Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration*

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STEM CELLS RESEARCH & THERAPY, 2015; 6:46

Wydajna regeneracja mięśni szkieletowych wymaga, aby komórki o potencjale miogenicznym były w stanie fuzjować ze sobą tworząc wielojądrowe miotuby, które dojrzewając odtwarzają uszkodzone włókna mięśniowe. Komórki mięśniowe wykazują zdolność do fuzji nie tylko *in vivo*, ale również w hodowli *in vitro*. Pozwala to w pewnym stopniu odwzorować pierwsze etapy różnicowania miogenicznego. Niezbędnymi czynnikami zaangażowanymi w fuzję komórek są białka adhezyjne. Badania prowadzone w Zakładzie Cytologii wskazywały na to, że mysie mioblasty stymulowane *in vitro* chemokiną Sdf-1 wykazują wyższy poziom białka adhezyjnego CD9. Również podanie Sdf-1 do regenerującego mięśnia szkieletowego myszy powoduje wzrost poziomu CD9.

Wiedząc o wpływie Sdf-1 na poziom CD9 chciałem sprawdzić, czy niesie to ze sobą efekt biologiczny i może wspomagać regenerację. W tym celu wykorzystałem mysie komórki macierzyste: ESC oraz BMSC, które stymulowałem Sdf-1 i sprawdzałem *in vitro*, czy spowoduje to wzrost ich zdolności do fuzji. W tym celu komórki macierzyste umieściłem w hodowli mieszanej razem z mioblastami. W trakcie takiej hodowli komórki mięśniowe fuzjują tworząc wielojądrowe miotuby. Jeżeli dodane komórki takie jak ESC lub BMSC, mają zdolność do fuzji z mioblastami, to część miotub będzie hybrydowa, tj. utworzona z ich udziałem. Częstość, z jaką powstają takie hybrydowe miotuby odzwierciedla potencjał badanych komórek do uczestniczenia w regeneracji mięśni szkieletowych.

Wykazałem, że BMSC stymulowane Sdf-1 zwiększały swoją zdolność do fuzji z mioblastami, co przekładało się na wyższy odsetek hybrydowych miotub. W przypadku ESC, które mają bardzo ograniczoną zdolność do fuzji z mioblastami, zaobserwowałem przede wszystkim zmianę w ich zdolności do migracji. ESC traktowane Sdf-1 zdecydowanie lepiej rozprzestrzeniały się w hodowli i zamiast tworzyć agregaty migrowały wzdłuż nowopowstających miotub. Wykorzystując technikę siRNA wyciszyłem w ESC ekspresję receptora Cxcr4. Dzięki temu wykazałem, że za wzrost poziomu CD9 po stymulacji Sdf-1 odpowiada aktywacja receptora Cxcr4.

Roztwór białka Sdf-1 podawałem również do regenerującego mięśnia myszy pozbawionej funkcjonalnych alleli genu Pax7. Dzięki temu mogłem zweryfikować, czy brak komórek satelitowych w mięśniu zmienia jego odpowiedź na Sdf-1. Wykazałem, że w odpowiedzi na Sdf-1 następuje wzrost poziomu CD9 w mięśniach myszy Pax7-/- w sposób podobny jak w mięśniach myszy kontrolnych.

Publikacja oryginalna 2, *Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration:*

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Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration

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Abstract

Introduction: Understanding the mechanism of stem cell mobilization into injured skeletal muscles is a prerequisite step for the development of muscle disease therapies. Many of the currently studied stem cell types present myogenic potential; however, when introduced either into the blood stream or directly into the tissue, they are not able to efficiently engraft injured muscle. For this reason their use in therapy is still limited. Previously, we have shown that stromal-derived factor-1 (Sdf-1) caused the mobilization of endogenous (not transplanted) stem cells into injured skeletal muscle improving regeneration. Here, we demonstrate that the beneficial effect of Sdf-1 relies on the upregulation of the tetraspanin CD9 expression in stem cells.

Methods: The expression pattern of adhesion proteins, including CD9, was analysed after Sdf-1 treatment during regeneration of rat skeletal muscles and mouse Pax7-/- skeletal muscles, that are characterized by the decreased number of satellite cells. Next, we examined the changes in CD9 level in satellite cells-derived myoblasts, bone marrow-derived mesenchymal stem cells, and embryonic stem cells after Sdf-1 treatment or silencing expression of CXCR4 and CXCR7. Finally, we examined the potential of stem cells to fuse with myoblasts after Sdf-1 treatment.

Results: *In vivo* analyses of *Pax7-/-* mice strongly suggest that Sdf-1-mediates increase in CD9 levels also in mobilized stem cells. In the absence of CXCR4 receptor the effect of Sdf-1 on CD9 expression is blocked. Next, *in vitro* studies show that Sdf-1 increases the level of CD9 not only in satellite cell-derived myoblasts but also in bone marrow derived mesenchymal stem cells, as well as embryonic stem cells. Importantly, the Sdf-1 treated cells migrate and fuse with myoblasts more effectively.

Conclusions: We suggest that Sdf-1 binding CXCR4 receptor improves skeletal muscle regeneration by upregulating expression of CD9 and thus, impacting at stem cells mobilization to the injured muscles.

Introduction

Skeletal muscle regeneration is a complex process of tissue degeneration and reconstruction [1]. The process mostly relies on the presence of muscle-specific unipotent stem cells; that is, satellite cells. However, the myogenic potential has also been shown for other populations of stem and progenitor cells [2]. Quiescent satellite cells that express transcription factor Pax7 are located between myofiber sarcolemma and basal lamina. In the response to muscle injury these cells are activated, begin to proliferate, differentiate into myoblasts, and fuse to form multinucleated myotubes and then muscle fibres. Satellite cell-derived myoblasts start to express myogenic regulatory factors responsible for their proper differentiation, such as Myod1, Myf5, Myf6, and myogenin [3]. The satellite cells, being muscle-specific stem cells, appear to be the cells of first choice to be tested in muscle therapies [4]. Nevertheless, for many reasons, their use is still limited. Among the major obstacles preventing the application of satellite cell-derived myoblasts in therapy, one can include their restricted ability to migrate through the vasculature to effectively engraft injured muscle, their rapid cell death after transplantation, and their limited regenerative capacity after *in vitro* culture [5].

Skeletal muscles serve as a niche not only for satellite cells but also for a few other populations of stem cells. These include muscle side population cells that were

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identified based on their ability to exclude Hoechst 33342 dye from their cytoplasm as well as the presence of stem cell antigen Sca1 and CD45 proteins [6]. In 2002 Asakura and Rudnicki demonstrated that these cells could fuse with myoblasts *in vitro* and also contribute to the formation of 1% of new myofibres when transplanted into the damaged anterior tibialis muscle of SCID mice [7]. Next, a small population (0.25%) of muscle side population-expressing satellite cell markers (that is, Pax7 and syndecan-4) as well as side population markers (that is, ATP-binding cassette subfamily member ABCG2 transport protein and stem cell antigen Sca1) participated in the formation of 30% of muscle fibres when transplanted into a damaged mouse anterior tibialis muscle and as many as 70% of the myofibres when transplanted into the anterior tibialis muscle of mdx mice [8]. Other populations of stem cells present within the skeletal muscle are pericytes associated with small blood vessels [9], mesangioblasts [10-13], AC133 stem cells that express CD133 [14], as well as $PW1^{+}/Pax7^{-}$ interstitial cells that synthesise PW1/PEG3 protein involved in tumour necrosis factor alpha–nuclear factor-κB signalling and do not express Pax7 protein [15]. These cells could undergo myogenic differentiation *in vitro* and *in vivo*; that is, after transplantation into regenerating mouse muscles. Furthermore, various tissues, including skeletal muscle, house multipotential mesenchymal stem cells (MSCs) that are defined based on adhesion to plastic, fibroblast-like morphology, intensive proliferation *in vitro*, and the ability to differentiate into adipocytes, chondrocytes, osteoblasts, and skeletal myoblasts [16,17]. MSCs isolated from mouse bone marrow improved muscle regeneration and also reduced fibrosis; that is, excessive development of connective tissue [18]. Moreover, MSCs isolated from the synovial membrane participated in the regeneration of mouse skeletal muscles and were present in the satellite cell niche. These cells also partially improved the function of skeletal muscle of mdx mice [17]. Additionally, many experiments showed that the regeneration of skeletal muscle can also be supported by stem cells isolated from tissues other than skeletal muscle. Among these were stem cells derived from bone marrow [19], human umbilical cord blood [20,21], human umbilical cord Wharton's jelly [22], and hematopoietic stem cells [23]. Furthermore, pluripotent stem cells such as embryonic stem cells (ESCs) [24,25] or induced pluripotent stem cells [26-28] could also follow a myogenic programme.

Even if many of the stem or progenitor cells manifest myogenic potential, they are rarely readily available for transplantation into injured muscle. First, transplantation of exogenous stem cells can be only effective when high doses of cells, ranging from $25 \times 10^6/cm^2$ to $67.5 \times 10^6/cm^2$ cells, could be administered directly into the muscle (100 injections per 1 cm^2) [29]. Second, transplanted cells are rarely able to migrate within injured muscle and for this reason they usually remain only at the site of the injection. Next, the method of cell administration can be also an issue. Despite their myogenic potential, many of the stem cells tested were not able to engraft injured muscle when transplanted into the bloodstream. This makes their use in therapy rather difficult and limited. Presently, the major limitations that contribute to the failure of clinical trials are caused by the lack of specific methods supporting homing of the stem cells after their systemic infusion. Summarising, comprehensive *in vitro* and *in vivo* studies demonstrated that many of stem cell populations are characterised by myogenic potential; that is, the ability to differentiate into myoblasts and muscle fibres and also to colonise the satellite cell niche. Next, the transplantation of these cells could improve regeneration of damaged muscles. However, their physiological role in the reconstruction of skeletal muscle remains unexplained.

In our previous study we showed that stromal-derived factor-1 (Sdf-1, also known as CXCL12) treatment improved skeletal muscle regeneration by enhancing endogenous (not transplanted) stem cell mobilisation into injured muscle [30]. Sdf-1 belongs to the cytokine family and acts on the cells expressing receptor CXC chemokine receptor (CXCR)-4 and/or CXCR7 [31]. Moreover, we were also analysing the role of various adhesion proteins in myoblast differentiation. M-cadherin [32], adhesion protein complex composed of ADAM-12, CD9, CD81, integrin beta1, and alpha3 [33], as well as syndecan-4 were shown by us to be engaged in myoblast differentiation [34]. Next, crucial function in this process of such proteins as integrin alpha7 [35], alpha9 [36], and other adhesion proteins was shown by other studies.

In the current study, we documented how Sdf-1 impacts on myoblasts and other stem cell properties, improving their ability to participate in the skeletal muscle regeneration. We also show that preconditioning of stem cells with Sdf-1 could be an effective approach to optimise stem cell migration and engraftment to injured muscles. Our results thus underline the mechanism that could be activated in order to mobilise endogenous cells into injured tissue. Importantly, this mechanism could also be switched on in order to enhance homing of the transplanted cells to the target tissues, and thus could allow reduction of the number of cells needed for the therapy.

Materials and methods

All procedures involving animals were approved by First Warsaw Local Ethics Committee for Animal Experimentation.

Cell cultures

Rat satellite cell-derived myoblasts

Slow twitch soleus muscles were dissected from the hind limbs of 3-month-old male WAG rats. The satellite cells were isolated by muscle digestion with 0.15% pronase (Sigma-Aldrich, St. Louis, MO, United States) in HAM's F-12 medium (Life Technologies, Carlsbad, California, United States) buffered with 10 mM HEPES (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS; Life Technologies). Cells were plated on 2% gelatine-coated (Sigma-Aldrich) dishes in complete Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS, 10% horse serum (Life Technologies), and 1% antibiotics (AB, 50 U/ml penicillin, 50 μg/ml streptomycin; Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide. The medium was changed every 2 days. Starting from day 3 of culture, cells were treated with 100 ng/ml Sdf-1. The control cells were cultured in the absence of Sdf-1 in the medium. The cells were subjected either to quantitative RT-PCR, immunolocalisation, or western blotting. The morphology of cells was analysed using a Nikon Eclipse TE200 microscope (Nikon Instruments, Tokyo, Japan) with Hoffman contrast.

C2C12 myoblasts

Mouse C2C12 myoblasts (obtained from the European Collection of Cell Cultures, Porton Down, United Kingdom) were plated in DMEM supplemented with 10% FBS and 1% AB, on 2% gelatine-coated plates. Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide. The morphology of cells was analysed using a Nikon Eclipse TE200 microscope with Hoffman contrast. The cells were subjected either to Sdf-1 treatment, transfection with small interfering RNA (siRNA) complementary to mRNA encoding CXCR4, quantitative RT-PCR, or immunolocalisation or used for co-culture experiments.

Mouse bone marrow-derived mesenchymal stem cells

The bones were dissected from the hind limbs of 3-month-old male C57Bl6N mice carrying the lacZ transgene in the ROSA26 locus. Next, the ends of the bones were cut and bone marrow was washed out with saline using a 22 G needle. Obtained cells were washed twice with saline. Erythrocytes were then removed by gradient centrifugation in Histopaque (Sigma-Aldrich) for 20 minutes at 1,800 rpm. Obtained mononucleate cells were separated using a magnetic column (MACS; Miltenyl Biotec, Bergisch Gladbach, Germany) with anti-CXCR4 specific antibody (Abcam, Cambridge, United Kingdom), according to the manufacturer's instruction. The $CXCR4^+$ fraction of cells was cultured in α-minimum essential medium (Sigma-Aldrich) supplemented with 20% FBS, 200 mM L-glutamine (Life Technologies), and 1% AB. The morphology of cells was analysed using a Nikon Eclipse TE200 microscope with Hoffman contrast. The cells were subjected either to Sdf-1 treatment, quantitative RT-PCR, or immunolocalisation or used for co-culture experiments.

Mouse embryonic stem cells

ESCs constitutively expressing histone H2B-GFP were provided by Dr Kat Hadjantonakis [37]. Mitomycin-inactivated mouse embryonic fibroblasts, which served as the feeder layer for ESCs, were plated on dishes coated with 1% gelatine (Sigma-Aldrich) in DMEM supplemented with 10% FBS and 1% AB. Twenty-four hours later ESCs were seeded onto the inactivated mouse embryonic fibroblasts and cultured in knockout DMEM (Life Technologies) supplemented with 15% ES-qualified FBS (Life Technologies), 0.1 mM nonessential amino acids (Sigma-Aldrich), 200 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1% AB, and 500 U/ml leukaemia inhibitory factor (Chemicon, Billerica, MA, United States). Prior to transfection, ESCs were separated from mouse embryonic fibroblasts by the preplating technique and cultured on dishes coated with Matrigel Matrix Growth Factor Reduced (1 mg/ml DMEM; BD Biosciences, Becton-Dickinson, San Jose, CA, United States). The morphology of cells was analysed using a Nikon Eclipse TE200 microscope with Hoffman contrast. The cells were subjected either to Sdf-1 treatment, transfection with siRNA complementary to mRNA encoding CXCR4 or CXCR7, quantitative RT-PCR, immunolocalisation, or western blotting or used for co-culture experiments.

Sdf-1 treatment and silencing of CXCR4 or CXCR7 expression by RNA interference

C2C12 or ESCs were plated on plates covered with Matrigel Matrix Growth Factor Reduced (BD Biosciences). After reaching 30 to 40% confluence the cells were transfected with Silencer Select Pre-designed siRNA (Life Technologies) complementary to mRNAs encoding either CXCR4 9 (ID:s64091) or CXCR7 (ID:s64124). Appropriate, recommended negative control siRNA was used. siRNA duplexes were diluted in DMEM to reach the concentration of 100 pmol per plate and incubated with Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's instructions. After 24 hours the cells were treated with Sdf-1 (10 ng/μl). Next, cells were collected 48 hours post Sdf-1 treatment and processed either for mRNA isolation followed by quantitative RT-PCR, immunolocalisation, or western blotting. The efficiency of CXCR4 or CXCR7 downregulation was tested by quantitative RT-PCR and immunocytochemistry.

Co-culture of stem cells and mouse C2C12 myoblasts

C2C12 myoblasts were plated at density of 3×10^4 in DMEM with 10% FBS and 1% AB. When C2C12 cells

reached confluence and started to fuse, bone marrow-derived mesenchymal stem cells (BM-MSCs) or ESCs – control or pretreated with Sdf-1 – were seeded. Respectively, 5×10^6 ESCs and 2×10^4 BM-MSCs were added. After 24 hours the medium was changed for differentiation; that is, DMEM supplemented with 3% horse serum and 1% AB. After 14 days of co-culture, cells were fixed in 3% paraformaldehyde (Sigma-Aldrich) and then processed for immunolocalisation of selected antigens, as described below. Skeletal myosin heavy and light chains (Sigma-Aldrich) were localised to define differentiated myotubes in ESC and myoblast co-cultures. β-galactosidase (Abcam) was localised to identified BM-MSCs in co-cultures with myoblasts. Cell nuclei were visualised with DraQ5 (Biostatus Limited, Biostatus Ltd, Leicestershire, United Kingdom) diluted in PBS. Cultures were analysed using confocal microscope Axiovert 100 M (Zeiss, Carl Zeiss Inc., Jena, Germany) and LSM 510 application software (Carl Zeiss Inc., Jena, Germany). The same image acquisition settings were used for all comparisons. For each experimental group, the number of hybrid myotubes was counted from 50 random fields of view. Data are the mean ± standard deviation of three biological replicates. Results were analysed by Student's *t* test using GraphPadPrism (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant when $P < 0.05$.

Migration assay

BM-MSCs or ESCs were plated into the inserts of six-well dishes (8 μm pores; BD Biosciences). Both inserts and wells were coated with Matrigel Matrix Growth Factor Reduced. After 24 hours of culture, the medium in the lower dish was replaced with the medium supplemented with 50 ng/ml Sdf-1. Control cells were cultured in medium lacking Sdf-1. After 48 hours of culture the cells were fixed and stained with Giemsa (Merck, Merck KGaA, Darmstadt, Germany). The number of cells that migrated from the inserts and localised either at the membrane surface facing the lower dish or at the bottom of the lower dish was counted. Three independent experiments were performed for each analysis. Data are the mean ± standard deviation of three biological replicates. Statistical analysis was performed with unpaired *t* test using GraphPadPrism (GraphPad Software, Inc.). The results were considered to be significantly different when $P < 0.05$.

Muscle injury

The regeneration of slow twitch soleus skeletal muscles was induced in 3-month-old male WAG rats. Briefly, the animals were anaesthetised with pentobarbital sodium salt (Sigma-Aldrich) by an intraperitoneal injection (30 mg/kg body mass). Next, muscles were exposed, denervated, and crushed as described previously [30]. Muscles were injected with 100 ng Sdf-1 diluted in 20 μl physiological saline. Two injections, 10 μl each, were administered into two different parts of muscle. The control muscles were injected with 20 μl physiological saline. The animals were euthanised with carbon dioxide at days 1, 3, and 7 after the muscle injury. Next, injured muscles were isolated, weighed, and collected for further analysis.

Quantitative RT-PCR

Total RNA was isolated from muscles, satellite cell-derived myoblasts, and C2C12 myoblasts using the High Pure Isolation Kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. RNA was extracted from biological replicates (two C2C12 cultures or three primary cell cultures or three muscle samples per each experimental time point). Then 100 ng RNA from each sample was reverse-transcribed using the RT^2 First Strand Kit (SABiosciences, Qiagen, Valencia, CA, United States) or the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol, for muscles and myoblasts, respectively. Next, mRNA levels in muscles and satellite cell-derived myoblasts were examined using a custom PCR array (SABiosciences) for the *genes* m-cadherin, ADAM12, syndecan-4, CD9, CD81, integrin beta1 (itgb1), alpha3 (itga3), alpha7 (itga7), and alpha9 (itga9) according to the manufacturer's protocol. mRNA levels in C2C12 myoblasts were examined using a custom PCR array based on Universal ProbeLibrary (Roche Applied Science) for the following *genes*: m-cadherin, ADAM12, CD9, CD81, itgb1, itga3, itga7, and itga9. Hypoxanthine phosphoribosyltransferase 1 (Hprt1) [RefSeq:NM_012583], glyceraldehyde-3-phosphate dehydrogenase (Gapdh) [RefSeq:NM_017008], beta-2-microglobulin (B2m) [RefSeq:NM_012512], and acidic ribosomal phosphoprotein P1 (Rplp1) [RefSeq:NM_001007604] were used as the candidate reference genes. Quantitative real-time PCR analyses were performed with the RT^2 Real-Time PCR Master Mix (SABiosciences) in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) or with the LightCycler 480 Probes Master 9.0 (Roche Applied Sciences) in the LightCycler 480 (Roche Applied Sciences), according to the PCR array manufacturer's instruction. Threshold cycle (Ct) values of the analysed amplicons were determined with SDS 2.1 software (Applied Biosystems) or LightCycler® 480 Software (Roche Applied Science). Expression levels were calculated with the $2^{-(\Delta CT)}$ formula using DataAssist™ software (Applied Biosystems) or the relative quantification tool in LightCycler® 480 Software. The geNorm™ algorithm integrated into DataAssist™ was used to evaluate the stability of the candidate reference genes. The expression level and standard deviation for each gene was represented as the column charts using GraphPadPrism. All the candidate reference genes (B2m, Gapdh, Hprt, and Rplp1) displayed high expression stability, as determined by the

geNorm tool [38], and therefore were used for the normalisation of the expression data. Data are the mean ± standard deviation of two (C2C12) or three biological replicates, each analysed in two technical replicates. Results were analysed by ratio paired *t* test and differences were considered statistically significant when $P < 0.05$.

Analyses of mRNA levels in BM-MSCs and ESCs included RNA isolation using the mirVana kit (Life Technologies) and then reverse transcription using Superscript (Life Technologies). The TaqMan assays (Life Technologies) and Master Mix (Life Technologies) were used to analyse the level of the genes CXCR4, CXCR7, and CD9 according to the PCR array manufacturer's instructions. Hprt1 was used as the reference gene. All reactions were performed in triplicates. The conditions of quantitative RT-PCR were as follows: reverse transcription, 25°C for 10 minutes, 42°C for 60 minutes, and 85°C for 5 minutes; quantitative PCR, template denaturation, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The collected data were analysed using LightCycler 96SW 1.1 software (Roche Applied Sciences). Data are the mean ± standard deviation of three biological replicates, each analysed in two technical replicates. Results were analysed by paired *t* test and differences were considered statistically significant when $P < 0.05$.

Immunocytochemistry

Selected antigens were immunolocalised in sections of regenerating muscles, as well as in *in vitro* cultured cells. Cells cultured were fixed with 3% paraformaldehyde for 10 minutes. Muscle cryosections were hydrated in PBS, fixed in 3% paraformaldehyde and washed with PBS. Next, sections or cells were permeabilised with 0.1% Triton X-100/PBS (Sigma-Aldrich), and incubated with 0.25% glycine (Sigma-Aldrich). Nonspecific binding of antibodies was blocked with 3% bovine serum albumin (Sigma-Aldrich) with 2% donkey serum (Sigma-Aldrich) for 1 hour. Samples were then incubated with primary antibodies diluted 1:100 in 3% bovine serum albumin overnight, washed with PBS, and incubated at room temperature with secondary antibodies diluted 1:200 in 3% bovine serum albumin for 1.5 hours. After washing with PBS, cell nuclei were visualised by incubation with DraQ5 (Biostatus Limited) diluted 1:1,000 in PBS for 10 minutes. Specimens were mounted with Fluorescent Mounting Medium (Dako Cytomation, Glostrup, Denmark). After the procedure was completed samples were analysed using the confocal microscope Axiovert 100 M (Zeiss) and LSM 510 software. The same image acquisition settings were used for all comparisons. The following primary antibodies were used: rabbit polyclonal anti-skeletal myosin (M7523; Sigma-Aldrich), rabbit polyclonal anti-β-galactosidase (ab12081; Abcam), mouse monoclonal anti-integrin alpha3 (sc-7019; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-integrin beta1 (sc-9936; Santa Cruz), rabbit polyclonal anti-ADAM12 (ab39155; Abcam), rabbit polyclonal anti-CD9 (C9993; Sigma-Aldrich), rabbit polyclonal anti-CXCR4 (ab2074; Abcam), rabbit polyclonal anti-CXCR7 (ab117836; Abcam), goat polyclonal anti-CD81 (sc-7102; Santa Cruz), mouse monoclonal anti-M-cadherin (ab78090; Abcam), and rabbit polyclonal anti-VCAM-1 (sc-8304; Santa Cruz). Secondary antibodies directed against mouse or rabbit primary antibodies conjugated with Alexa488, Alexa594, and Alexa633 were used (A21202, A11059, A21206, A11034, A11080, A21203, A11037, A21071, A21082, A21063; Life Technologies). Appropriate controls of secondary antibodies were performed.

Western blotting

Fifty micrograms of total protein lysate were denatured by boiling in Laemmli buffer, separated using SDS-PAGE electrophoresis, and transferred to polyvinylidene fluoride membranes (Roche Applied Science). The membranes were washed, blocked with 5% Blotto (BioRad, Bio-Rad, Hercules, CA, USA) and Tris-buffered saline for 1 hour, and incubated at 4°C with primary antibodies diluted 1:2,000 in 5% Blotto (BioRad) and Tris-buffered saline overnight, followed by secondary antibodies diluted 1:20,000 for 2 hours. Next, protein bands were visualised with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Langenselbold, Germany) and exposed to chemiluminescence positive film (Amersham Hyperfilm ECL; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The obtained results were analysed with GelDoc2000 using Quantity One software (BioRad). Primary antibodies used were mouse monoclonal anti-integrin alpha3 (sc-7019; Santa Cruz), rabbit polyclonal anti-integrin beta1 (sc-9936; Santa Cruz), rabbit polyclonal anti-ADAM12 (ab39155; Abcam), rabbit polyclonal anti-CD9 (C9993; Sigma-Aldrich), goat polyclonal anti-CD81 (sc-7102; Santa Cruz), rabbit polyclonal anti-CXCR4 (ab2074; Abcam), rabbit polyclonal anti-CXCR7 (ab117836; Abcam), rabbit polyclonal anti-M-cadherin (sc-10734; Santa Cruz), rabbit polyclonal anti-VCAM-1 (sc-8304; Santa Cruz), and mouse monoclonal anti-tubulin (T5168; Sigma-Aldrich). Secondary antibodies used were peroxidase-conjugate rabbit anti-mouse (A9044; Sigma-Aldrich), peroxidase-conjugate rabbit anti-goat (A5420; Sigma-Aldrich), and peroxidase-conjugate goat anti-rabbit (A9169; Sigma-Aldrich). Three independent experiments were performed.

Results

Sdf-1 treatment changes expression of adhesion proteins during myoblast differentiation *in vitro* and *in vivo* in regenerating muscle

In our previous studies we evidenced that Sdf-1 improved muscle regeneration, stem cell mobilisation, and

myoblast migration [30]. Since adhesion proteins play a crucial role in the myogenic processes we decided to focus on the possible link between Sdf-1 and those proteins engaged in myoblast migration and differentiation. To verify the existence of such a link we first focused on skeletal muscle regeneration.

To follow the impact of Sdf-1 on regeneration, soleus muscles of WAG rats were injected with Sdf-1 (100 ng per muscle) after the muscle injury. Next, we analysed nontreated (control) and Sdf-1-treated muscles at days 1 and 3 of regeneration (Figure 1). Activated satellite cells start to proliferate (day 1), differentiate into myoblasts (day 3) that fuse (day 7) to form myotubes, and reconstruct damaged myofibres. The levels of mRNAs encoding adhesion proteins (that is, i.e. m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin beta1, alpha3, alpha7, and alpha9) were compared between control and Sdf-1-treated muscles, at days 1 and 3 of regeneration. At day 3, Sdf-1 significantly increased expression of m-cadherin, ADAM-12, and integrin alpha9 at the mRNA level (Figure 1A). Changes in mRNA levels was readily translated to the levels of m-cadherin, ADAM-12, and integrin alpha9 proteins, which dramatically increased in mononucleated cells present within the regenerating muscle (day 3), as shown by immunolocalisation (Figure 1B). We did not observe significant changes in the mRNAs encoding other analysed factors; that is, syndecan-4, CD9, CD81, integrin beta1, alpha3, and alpha7. However, immunolocalisation revealed that Sdf-1 impacted one of the tetraspanins (that is, CD9). Immunolocalisation of CD9 showed that this tetraspanin was present in mononucleated cells both in control and Sdf-1-treated muscles at day 3 of regeneration of the soleus muscle (Figure 1B). At day 7 of regeneration, CD9 was still detectable in mononucleated cells and rarely in newly formed myofibres in control muscles (Figure 1C). However, in Sdf-1-treated muscles this protein was detectable in mononucleated cells and significantly in the cell membranes of newly formed myofibres (Figure 1C). Western blotting also showed the changes at the protein level after Sdf-1 treatment (Figure 1D). The level of m-cadherin, itga9, and CD9 increased at days 1 and 3 of muscle regeneration in response to Sdf-1 treatment. The changes in

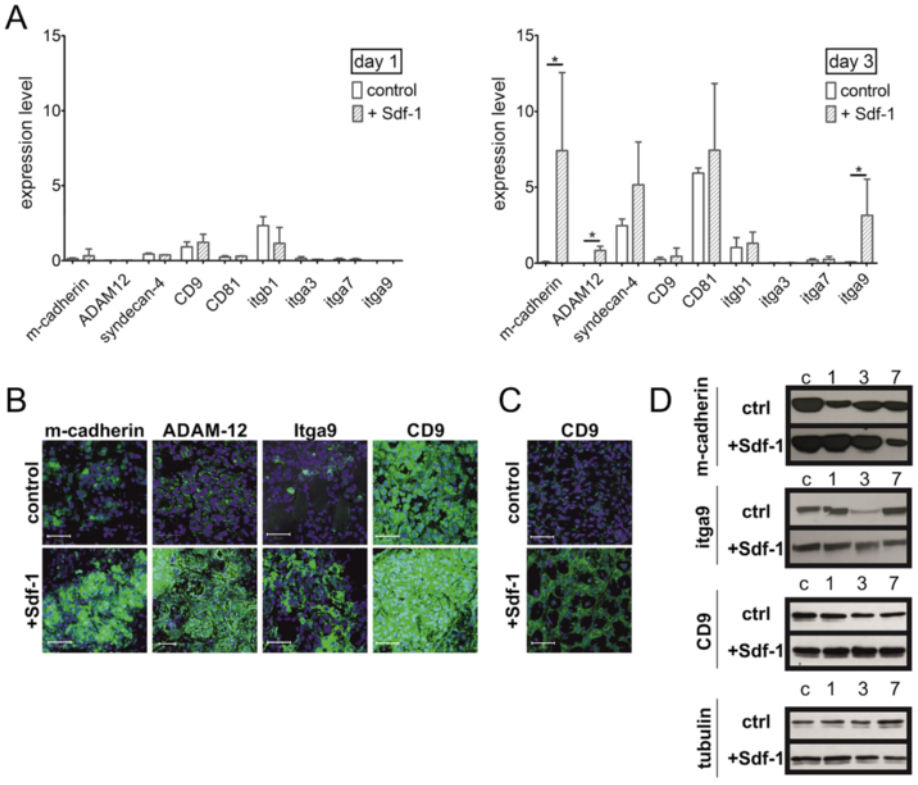

Figure 1 Sdf-1 impact on the expression of adhesion proteins in regenerating rat soleus muscle. (A) Quantitative RT-PCR analysis of mRNAs encoding m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin β1 (itgb1), integrin α3 (itga3), integrin α7 (itga7), and integrin α9 (itga9) in control and Sdf-1-treated muscles (Sdf-1) at days 1 and 3 of regeneration. **(B)** Immunolocalisation of m-cadherin, ADAM-12, itga9, and CD9 in control and Sdf-1-treated muscles at day 3 of regeneration. Bar = 50 μm. **(C)** Immunolocalisation of CD9 in control and Sdf-1-treated muscles at day 7 of regeneration. **(D)** Level of m-cadherin, itga9, and CD9 protein during control (ctrl) and Sdf-1-treated muscle regeneration at days 1, 3, and 7 (C – intact muscle). *P <0.05. Error bars indicate standard deviation.

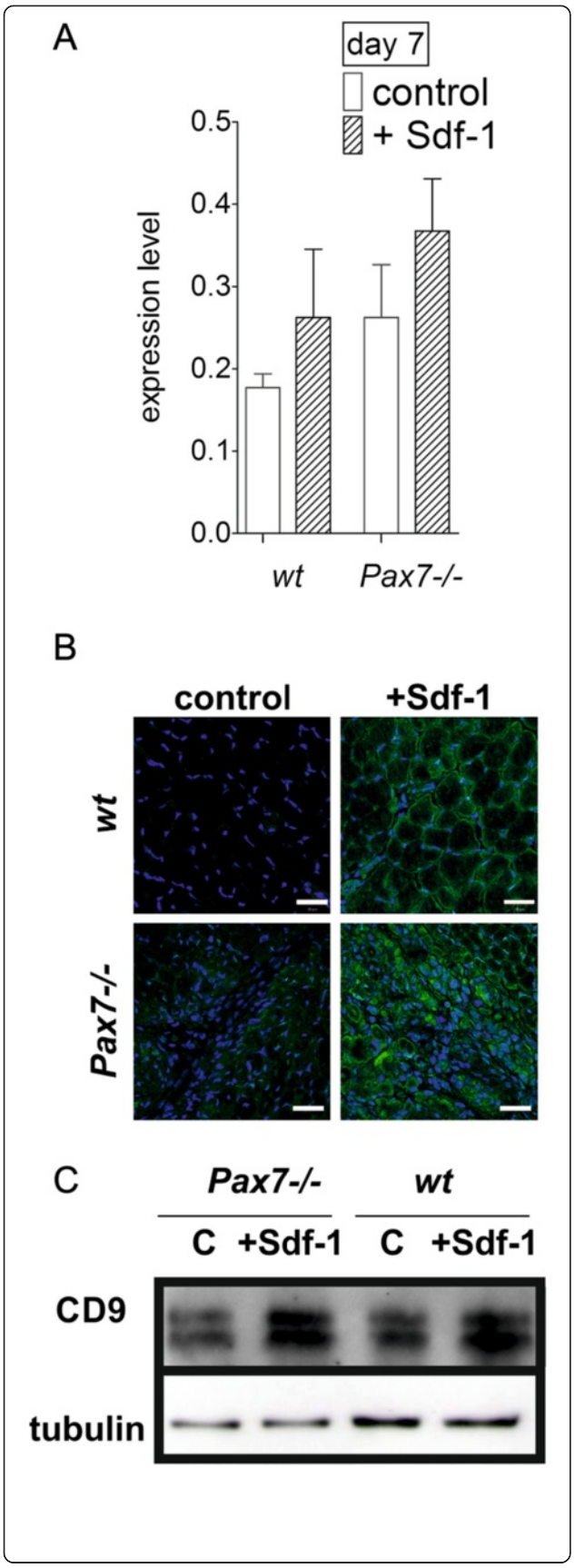

Figure 2 CD9 in control and injected with Sdf-1 skeletal muscles of wild type and *Pax7*$^{-/-}$ mice. (A) Quantitative RT-PCR analysis of mRNA encoding CD9 in wild type (*wt*) and *Pax7*$^{-/-}$ mice muscles at day 7 of regeneration. **(B)** Immunolocalisation of CD9 in *wt* and *Pax7*$^{-/-}$ mouse muscles at day 7 of regeneration. Nuclei, blue; adhesion proteins, green. Bar = 30 μm. **(C)** Level of CD9 protein during control (C) and Sdf-1-treated muscle regeneration of *wt* and *Pax7*$^{-/-}$ mice at day 7. Error bars indicate standard deviation.

CD9 level were also detectable at day 7 (Figure 1D). Importantly, as we showed previously, Sdf-1 did not change the number of rat satellite cell-derived myoblasts during *in vitro* culture, implying that it did not impact the proliferation rate [30].

To determine whether Sdf-1 increases CD9 expression in satellite cell-derived myoblasts or impacts on cells migrating to the injured muscle, we decided to focus on *Pax7*$^{-/-}$ mice. Previous analyses of this mouse model did not show any abnormalities in embryonic myogenesis [39]. However, postnatal development in *Pax7*$^{-/-}$ mice is associated with a dramatic decrease in the population of satellite cells, which causes the muscle growth retardation [40]. As a result these mice are significantly smaller than wild-type (*wt*) mice, have difficulty in moving, and usually die within 3 weeks of age. Analyses of *Pax7*$^{-/-}$ muscles give us a unique opportunity to answer the question about the identity of cells upregulating CD9 within the injured muscle; that is, we were able to test whether Sdf-1 treatment impacted on the resident satellite cells (absent in *Pax7*$^{-/-}$ mice) or the cells that were infiltrating injured muscle. Again, Sdf-1 treatment increased the CD9 mRNA level only slightly (Figure 2A). However, the level of CD9 protein was higher in *Pax7*$^{-/-}$ and *wt* mice muscles as showed by immunocytochemistry and western blotting (Figure 2B,C). CD9 protein exists in three forms with molecular masses between 22 and 27 kDa, and thus two CD9 bands were detected by western blot. Summarising, we proved that Sdf-1 injected into the muscle upregulated the CD9 level also in cells other than the satellite cells that either are already present within or infiltrate regenerating muscle. To answer the question of whether Sdf-1 also acts at satellite cells, we turn to the *in vitro* system.

The notion that Sdf-1 treatment results in upregulation of CD9 in satellite cells was tested in *in vitro* experiments in which we took advantage of primary rat satellite cell-derived myoblasts. At day 5 of culture, satellite cell-derived myoblasts start to proliferate and then, at day 7, fuse to form multinucleated myotubes. We did not observe any significant differences in m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin beta1, alpha3, alpha7, and alpha9 mRNA levels between control and Sdf-1-treated cells at day 5 (nondifferentiated cells) and day 7 of culture (fusing cells) (Figure 3A). When

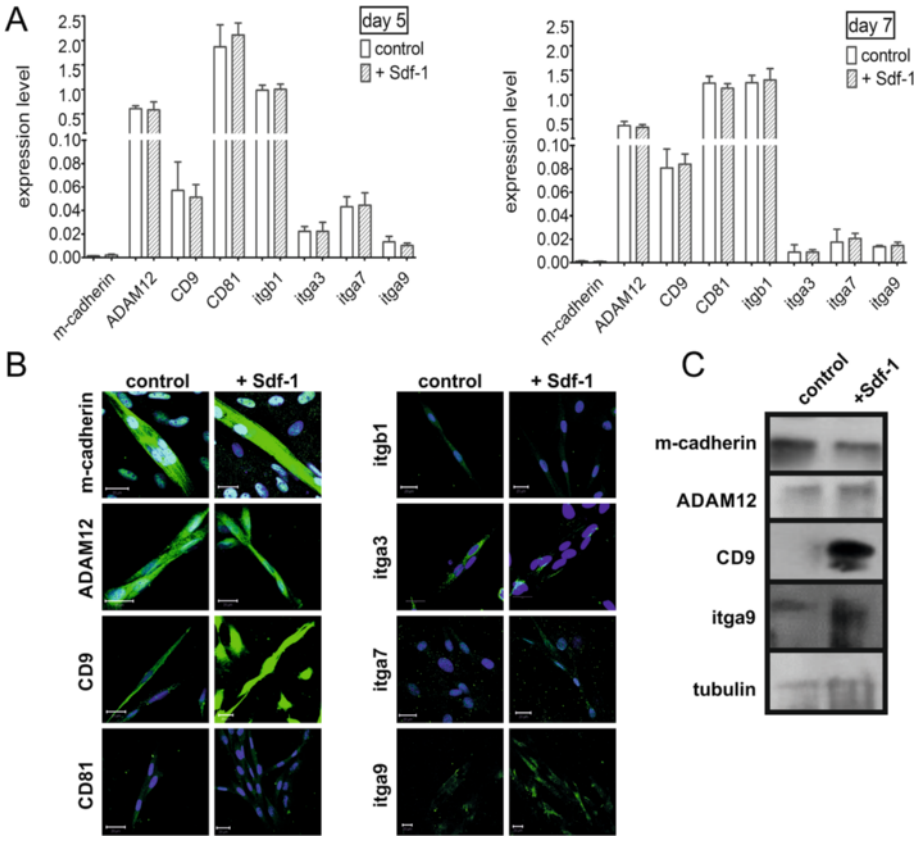

Figure 3 Sdf-1 impacts on the expression of adhesion proteins (m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin β1, integrin α3, integrin α7, integrin α9) in differentiating rat satellite cell-derived myoblasts. (A) Level of mRNA at days 5 and 7 of control (C) and Sdf-1-treated myoblast (Sdf-1) differentiation. **(B)** Immunolocalisation of adhesion proteins in control and Sdf-1-treated cells. Nuclei, blue; adhesion proteins, green. Bar = 20 μm. **(C)** Level of adhesion proteins during control and Sdf-1-treated myoblast differentiation at day 7. itgb1, integrin β1; itga3, integrin α3; itga7, integrin α7; itga9, integrin α9. *P <0.05. Error bars indicate standard deviation.

assessing the levels of the proteins by immunolocalisation and western blotting we observed a spectacular increase only in the case of CD9 (Figure 3B,C).

Downregulation of Sdf-1 receptor (CXCR4) affects CD9 expression in C2C12 myoblasts

To further dissect the Sdf-1 impact on myoblast differentiation we decided to manipulate the levels of its receptor; that is, CXCR4. In these experiments we used mouse C2C12 myoblasts. The rationale behind the choice of these cells is based on the fact that this cell line not only serves as a standard in the studies on myoblast differentiation, but also is easy to manipulate and transfect in *in vitro* culture. Expression of CXCR4 was downregulated with specific siRNA. Forty-eight hours following transfection with siRNA the level of CXCR4 mRNA decreased to 42.72 ± 2.39%, as compared with cells transfected with control siRNA cells. In addition, the level of CXCR7 mRNA, which was shown to be involved in the Sdf-1 and interferon-inducible T-cell chemoattractant signalling pathway [41], also slightly decreased to 77.14 ± 8.39%. Downregulation of CXCR4 did not change significantly the levels of mRNAs encoding adhesion proteins; that is, m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin beta1, alpha3, and alpha7 (Figure 4A). Moreover, Sdf-1 treatment did not impact on the levels of analysed mRNAs (Figure 4A). However, the difference in ADAM12 mRNA level after silencing CXCR4 expression and Sdf-1 treatment was statistically significant ($P = 0.046$), but it was not statistically significant when compared with control. Next, the expression of itga9 mRNA was very low and changed neither after Sdf-1 treatment nor after downregulation of CXCR4 (data not shown). The downregulation of CXCR4 level was translated to the protein level (Figure 4B). Moreover, the level of CD9 protein increased after Sdf-1 treatment and decreased in response to CXCR4 downregulation (Figure 4B). Immunolocalisation again proved that CXCR4 protein was not detectable in siRNA transfected cells (Figure 4C). We also did not notice changes in the localisation and levels of m-cadherin, ADAM-12, syndecan-4, CD81, integrin beta1, alpha3, alpha7, and alpha9

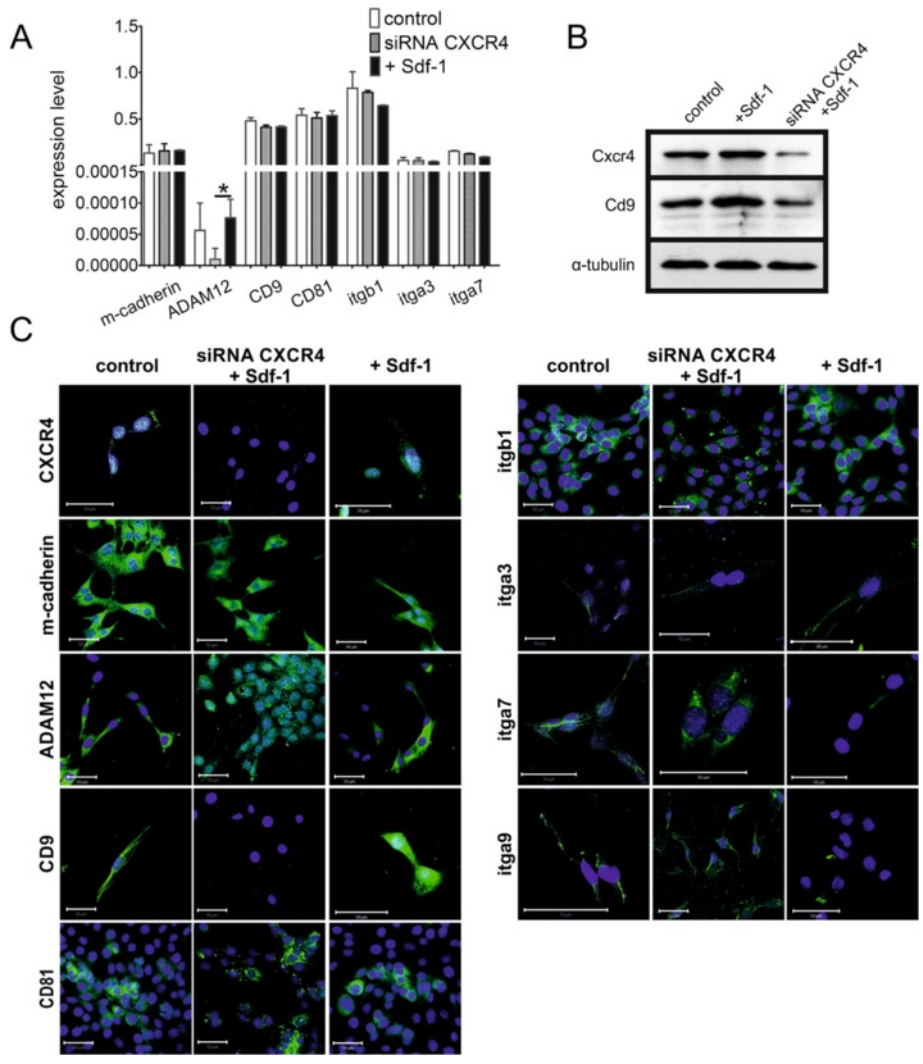

Figure 4 Level of CXCR4 and adhesion proteins in C2C12 myoblasts. (A) Quantitative RT-PCR analysis of mRNA encoding m-cadherin, ADAM-12, syndecan-4, CD9, CD81, integrin β1 (itgb1), integrin α3 (itga3), integrin α7 (itga7), and integrin α9 (itga9) in control cells, treated with Sdf-1, and transfected with CXCR4 siRNA (siRNA). **(B)** Western blotting analysis of CXCR4, CD9, and tubulin in control, Sdf-1-treated (Sdf-1) and transfected with CXCR4 siRNA (siRNA) C2C12 myoblasts. **(C)** Immunolocalisation of CXCR4 and adhesion protein in control, treated with Sdf-1, and transfected with CXCR4 siRNA (siRNA) C2C12 myoblasts. Nuclei, blue; adhesion proteins, green. Bar = 50 μm. CXCR, CXC chemokine receptor; siRNA, small interfering RNA. *P <0.05. Error bars indicate standard deviation.

(Figure 4C). Importantly, CXCR4 silencing abolished Sdf-1 induced CD9 upregulation (Figure 4C).

Sdf-1 upregulates CD9 expression in bone marrow-derived stem cells and embryonic stem cells

Our next question was whether Sdf-1 affected CD9 expression in stem cells other than satellite cells or cells infiltrating the injured skeletal muscle. To address this issue we decided to analyse two standard stem cell types of different origin: multipotent adult BM-MSCs and pluripotent ESCs. Both types of cells are extensively studied as a source of cells that could be used in therapy.

Cells isolated from mouse bone marrow were separated using a magnetic column and the fraction of CXCR4-positive cells (BM-MSCs^{CXCR4+}) – that is, only the cells able to react to Sdf-1 – were analysed. We showed that the level of CXCR4 protein is higher in BM-MSCs^{CXCR4+} than in BM-MSCs^{CXCR4-} or the whole population of BM-MSCs (Figure 5A). Importantly, Sdf-1 treatment lead to the significant increase of CD9 mRNA and protein levels in BM-MSCs^{CXCR4+} (Figure 5B,C). CD9 protein exists in three forms with molecular masses between 22 and 27 kDa, and thus two CD9 bands were detected by western blot. Next, we tested the impact of Sdf-1 on ESCs, control and transfected with siRNA against CXCR4 or CXCR7. The mRNA and protein level

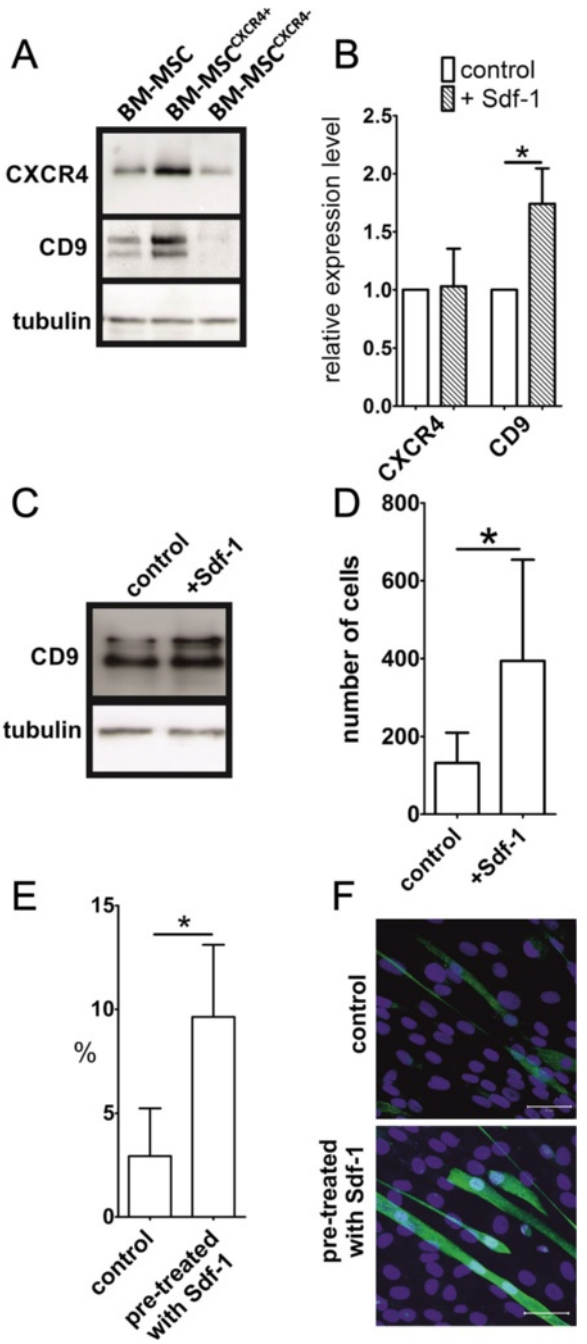

Figure 5 Sdf-1 impact on bone marrow mesenchymal stem cells. (A) Western blotting analysis of CXCR4, CD9, and tubulin in the whole population of bone marrow-derived mesenchymal stem cells (BM-MSCs) as well as of $CXCR4^+$ and $CXCR4^-$ BM-MSCs fractions fractions. **(B)** Quantitative RT-PCR analysis of CXCR4 and CD9 mRNA in BM-MSCs in control and Sdf-1-treated BM-MSCs. **(C)** Western blotting analysis of CD9 and tubulin in control and Sdf-1-treated (Sdf-1) BM-MSCs. **(D)** Migration of BM-MSCs in Sdf-1 gradient. The number of cells that migrated from the inserts was counted. **(E)** Percent of hybrid myotubes formed in co-culture of C2C12 myoblasts and control or Sdf-1 pretreated BM-MSCs. **(F)** Co-culture of C2C12 myoblasts and control or Sdf-1 pretreated BM-MSCs (green, localisation of β-galactosidase; blue, nuclei). Bar = 50 μm. CXCR, CXC chemokine receptor. *P <0.05. Error bars indicate standard deviation.

of CXCR4 did not change after Sdf-1 treatment and the level of CXCR4 protein was significantly downregulated in cells transfected with CXCR4 siRNA (Figure 6A,B). Notably, in the response to Sdf-1, ESCs also upregulated CD9 at the mRNA and protein levels (Figure 6A,B). Silencing of CXCR4 lead to the downregulation of CD9 protein in ESCs (Figure 6B). Downregulation of CXCR4 did not change the protein level of the second Sdf-1 receptor; that is, CXCR7 (Figure 6B). We also decided to silence expression of CXCR7 and observed that this only slightly reduced the CD9 protein level (Figure 6B). However, silencing of CXCR7 expression was connected with lower CXCR4 expression. We thus concluded that observed lower CD9 protein expression could be the result of CXCR4 downregulation.

Stem cells treated with Sdf-1 migrate and fuse with myoblast more effectively than control cells

Having found that Sdf-1, acting via CXCR4, upregulates CD9 in myoblasts, cells infiltrating injured muscle, and in such stem cells as BM-MSCs and ESCs, we decided to assess whether this phenomenon contributes to the improvement of skeletal muscle regeneration. We used *in vitro* systems allowing assessment of the cell migration ratio and the myogenic potential of analysed cells.

First, the migration of BM-MSCs and ESCs in response to Sdf-1 was analysed. Using the transwell migration system we showed that the number of BM-MSCs which migrated in response to Sdf-1 increased 3.0 times (Figure 5D). The number of ESCs that migrated increased 3.25 times in the presence of Sdf-1 (Figure 6C). Silencing of CXCR4, but not CXCR7, expression lead to the decrease of ESC migration in response to Sdf-1 treatment (Figure 6C). Next, we analysed how Sdf-1 impacts on the myogenic potential of BM-MSCs or ESCs. To this point, we co-cultured cells pretreated with Sdf-1 with differentiating C2C12 myoblasts. Analysis of co-culture of BM-MSCs with C2C12 cells revealed that they were able to form 2.93 ± 2.3% of hybrid myotubes; that is, tubules formed as a result of fusion between tested stem cells and C2C12 myoblasts. Sdf-1 pretreatment increased this number to 9.63 ± 3.5% (Figure 5E,F). Control ESCs were able to form 0.41 ± 0.38% of hybrid myotubes. In response to Sdf-1 pretreatment, the number of hybrid myotubes increased to 1.13 ± 0.64% (Figure 6D,E). As we have shown previously, ESCs very rarely fuse with myoblasts [42]. The observed increase in the fusion index after Sdf-1 treatment was thus an interesting result. Taken together, our results indicate that Sdf-1 upregulated CD9 expression in a CXCR4-dependent, but not a CXCR7-dependent, manner, induced stem cell migration, and increased myogenic potential of analysed stem cells.

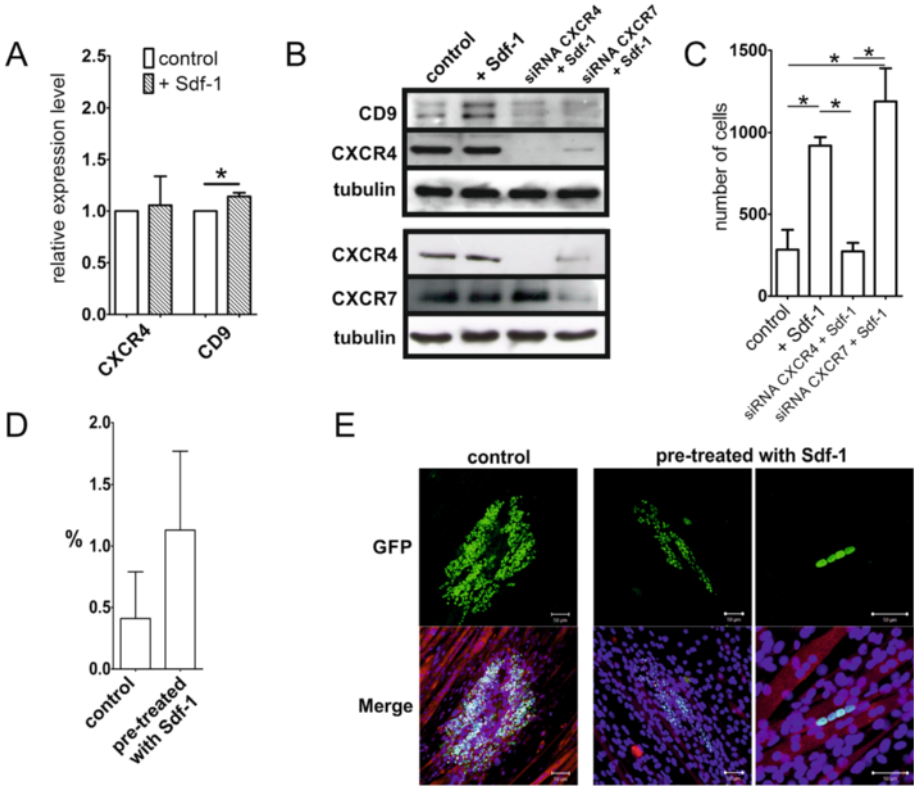

Figure 6 Sdf-1 impact on embryonic stem cells. (A) Quantitative RT-PCR analysis of CXCR4 and CD9 mRNA in control and Sdf-1-treated embryonic stem cells (ESCs). **(B)** Western blotting analysis of CXCR4, CXCR7, CD9, and tubulin in control, Sdf-1-treated (Sdf-1), and either CXCR4 (siRNA CXCR4) or CXCR7 siRNA-treated (siRNA CXCR7) ESCs. **(C)** Migration of control or transfected with CXCR4 or CXCR7 siRNA ESCs in response to Sdf-1 gradient. **(D)** Proportion of hybrid myotubes formed in co-culture of C2C12 myoblasts and control or Sdf-1 pretreated ESCs. **(E)** Co-culture of C2C12 myoblasts (red) and control or Sdf-1 pretreated ESCs (green); nuclei, blue. Bar = 50 μm. CXCR, CXC chemokine receptor. *P <0.05. Error bars indicate standard deviation.

Discussion

Previously, we showed that Sdf-1 improved regeneration of injured skeletal muscles by inducing stem cell mobilisation to injured muscle and also increasing myoblast migration via matrix metalloproteinases MMP2 and MMP9 [30]. However, Sdf-1 did not change the expression of myogenic regulatory factors either *in vivo* or *in vitro* [30]. Next, many lines of evidence, including our own studies, showed that adhesion proteins play a crucial and indispensable role in skeletal muscle regeneration [32,33,43,44]. Thus, in the current work we tested whether and how Sdf-1 affects expression of adhesion proteins engaged in myoblast migration and differentiation. We found that the levels of adhesion proteins increased in Sdf-1-treated muscles but not in *in vitro* cultured myoblasts (primary culture or cell line). This led us to the suggestion that *in vivo* the increase of m-cadherin, integrin alpha9, and ADAM12 could occur in cells other than myoblasts engaged in the regeneration of skeletal muscle. Nevertheless, our most important observation was that Sdf-1 induced upregulation of CD9 *in vivo* during *wt* and $Pax7^{-/-}$ muscle regeneration and in *in vitro* cultured myoblasts and such stem cells as BMSCs and ESCs.

CD9 is a 21 to 24 kDa surface molecule that belongs to the tetraspanins, a family of four-transmembrane domain proteins associated with integrin receptors, which was described as motility-related factor engaged in migration of multiple cancer cell lines [45]. CD9 was also shown to be associated with such integrins as α3β1, α4β1, α5β1, α6β1, α6β4, and αIIbβ3 [45]. Interactions of CD9 with integrins led to changes in their conformation and activation, which results in the modulation of integrin-dependent signalling pathways [46]. Moreover, CD9 is directly associated with EWI-2 and CD9P-1 (also known as EWI-F or FPRP, member of the immunoglobulin superfamily), epidermal growth factor receptor, and discodin domain receptor DDR1 [47-49]. Additionally, the tetraspanin network modulates membrane-type 1 matrix metalloproteinase cell surface localisation and is able to induce expression and also activate MMP2 [50,51]. By impacting at matrix metalloproteinases, CD9 may regulate not only cell migration but also tissue remodelling during embryonic development, angiogenesis,

tumour invasion and metastasis, and also tissue regeneration. Importantly, CD9 was also shown to play a role in muscle fibre formation [52]. In 1999 Tachibana and Hemler documented that anti-CD9 antibodies inhibited fusion of mouse C2C12 myoblasts, without affecting muscle-specific protein expression such as myosin heavy chains, desmin, and actin [52]. In our previous study, we also showed that the complex of CD9 and integrin α3β1 plays a pivotal role during satellite cell-derived myoblast fusion and skeletal muscle regeneration [33]. Interestingly, β1-deficient myoblasts that were unable to fuse did not express CD9 [53]. Moreover, Charrin and coworkers showed that proper muscle regeneration required CD9 and CD81 function [54]. They demonstrated that mice lacking either CD9 or CD81, or both CD9 and CD81, were unable to properly regenerate their skeletal muscles. During reconstruction of CD9 and CD81-deficient muscle, myoblasts formed giant dystrophic myofibres. Also *in vitro* absence of both CD9 and CD81 led to hyperfusion of myoblasts. Myoblasts lacking either CD9 or CD81 fused *in vitro* normally.

If a lack of CD9 decreases cell fusion, then its upregulation should have the opposite effect. Indeed, human rhabdomyosarcoma-derived myoblasts overexpressing CD9 formed approximately fourfold more syncytia than control cells [52]. In the current study we showed that Sdf-1 seems to be a perfect trigger leading to the increase in the CD9 proteins levels that promotes skeletal muscle regeneration via induction of stem cell migration and fusion with myoblasts. First, we noticed that Sdf-1 treatment results in upregulation of CD9 in myoblasts in a CXCR4-dependent way. Next, using *Pax7*$^{-/-}$ mice, we showed that Sdf-1 treatment also increased CD9 expression in cells other than satellite cells and differentiating myoblasts; that is, stem cells that infiltrate regenerating muscles. Our *in vitro* studies focusing on BM-MSCs and ESCs proved that stem cells are prone to Sdf-1/CXCR4-dependent CD9 induction, which leads to their increased migration and ability to fuse with myoblasts. Thus, we suggest that preconditioning of stem cells with Sdf-1 could be an alternative approach to optimise stem cell migration and engraftment after their injection into injured skeletal muscle. Presently, the major limitation causing the failure of clinical trials is the lack of specific homing of cells transplanted into injured tissue [55]. Some evidence shows that Sdf-1 treatment could be a strategy to improve the therapeutic potential of stem cells [56]. Sdf-1 treatment of endothelial progenitor cells improved their migration and adhesion to activated endothelium [57]. Sdf-1-treated endothelial progenitor cells from human umbilical cord or cord blood upregulated expression of integrins (α4 and αM) and MMP2 secretion [57]. Moreover, Sdf-1-treated mesangioblasts migrated more effectively *in vitro*, and *in vivo* efficiently engrafted mouse dystrophic muscles improving the reconstruction of muscle fibres [58]. BM-MSCs preconditioning with Sdf-1 increased cell viability, proliferation, and vascular endothelial growth factor secretion *in vitro* [59]. Sdf-1 was also shown to promote homing and proliferation of transplanted MSCs into infarcted myocardium [59]. Importantly, rat hearts transplanted with Sdf-1-pretreated MSCs showed significant neoangionesis in the ischaemic area [59].

The therapeutic potential of MSCs such as BM-MSCs is extensively explored. MSCs can be easily isolated from adult tissues and cultured *in vitro*. Notably, these cells exhibit no significant immunogenicity [60,61] and are able to differentiate into various cell types, producing

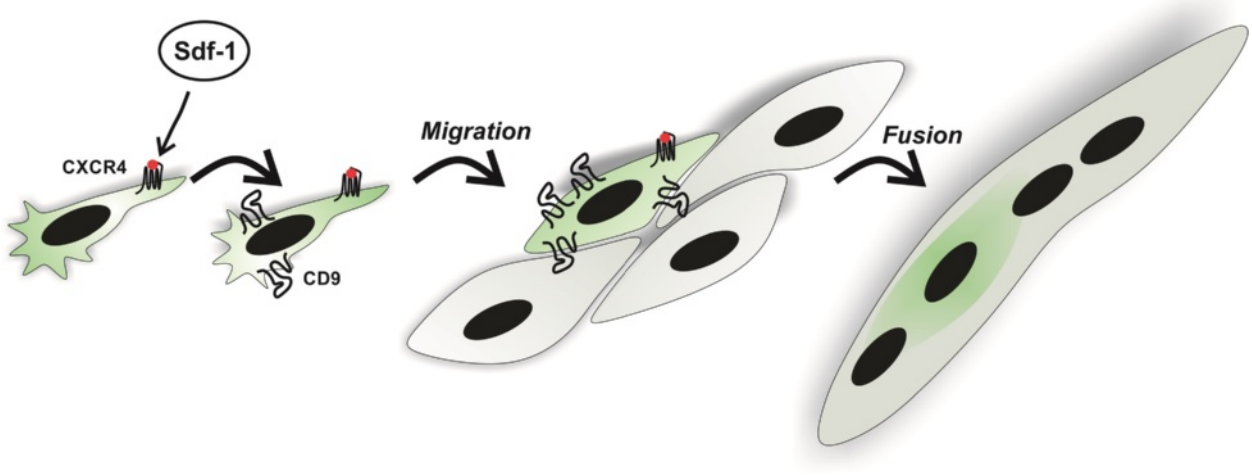

Figure 7 Myogenic potential of stem cells is not sufficient to apply them in damaged skeletal muscle therapy. Induction of CD9 expression in a Sdf-1 CXCR4-dependent way leads to increased ability of stem cell lines to migrate and fuse with myoblasts. CXCR, CXC chemokine receptor.

cytokines and growth factors characterised by anti-apoptotic, anti-inflammatory, and pro-angiogenic properties [62]. MSCs are also able to effectively follow the myogenic programme [17]. On the other hand, ESCs that are characterised by the potential to differentiate *in vivo* into any given cell type fail to efficiently produce some cell types *in vitro*. Myogenic differentiation of ESCs does not occur spontaneously even in embryonic bodies that mimic spatiotemporally early embryonic development [63,64]. As was shown by Darabi and co-workers, ESC overexpression of *Pax3* or *Pax7* can effectively drive the cells into a myogenic programme [25,26,65]. Other *in vitro* methods, such as culture conditions [66] or various chemical treatments [67], are far less effective. Here, we showed that upregulation of CD9, as the result of Sdf-1 pretreatment, leads to the increase in ability to migrate and fuse with myoblasts of these two stem cell lines; that is, BM-MSCs and ESCs.

The mechanism of CD9 expression is particularly interesting. It is known that CD9 mRNA exists in two major RNA species differing only in the length of their 5′ untranslated region [68]. Efficient mRNA translation depends, among other factors, on the supportive RNA folding of the 5′ untranslated region; that is, the region which contains the initiation codons. The long and short forms of the 5′ untranslated region of CD9 mRNA have different stability. The long 5′ untranslated region is characterised by a complex secondary structure comprising a stable stem-loop. A shift from shorter to longer 5′ untranslated regions influences the CD9 protein level. Thus, not only a reduction or increase in the absolute quantity of CD9 mRNA can reduce or increase the level of CD9 protein. Moreover, the mechanism of CD9 upregulation is concerned with some suggestions coming from the study, which showed in human $CD34^+$ cells isolated from cord blood that Sdf-1 acting through CXCR4 induced expression of CD9 via G-proteins and kinases they activate – protein kinase C, phospholipase C, extracellular signal-regulated kinase, and Janus kinase 2 signals [69]. Furthermore, pretreatment of human $CD34^+$ cells with anti-CD9 antibody significantly impaired their spleen and bone marrow homing [69].

Conclusions

The myogenic potential of stem cells is not sufficient to apply the cells in damaged skeletal muscle therapy. Here, we have shown that Sdf-1, through the CXCR4 receptor, induced expression of tetraspanin CD9 in satellite cell-derived myoblasts, ESCs, and BM-MSCs. We showed that upregulation of CD9 led to an increase in the ability of stem cells lines to migrate and fuse with myoblasts (Figure 7). Induction of CD9 could thus increase the therapeutic potential of stem cells.

Abbreviations

AB: antibiotics; BM-MSC: bone marrow-derived mesenchymal stem cell; CXCR: CXC chemokine receptor; DMEM: Dulbecco's modified Eagle's medium; ESC: embryonic stem cell; FBS: foetal bovine serum; MSC: mesenchymal stem cell; Sdf-1: stromal-derived factor-1; siRNA: small interfering RNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EB designed the study, performed molecular studies and immunoassays (rat satellite cell-derived myoblasts), and drafted the manuscript. KK carried out the molecular studies, western blot analysis, and immunoassays (Pax7 mice, ESCs, BM-MSCs), and participated in manuscript preparation. AM-Z carried out the molecular studies, western blot analysis, and immunoassays (skeletal muscles). MK and RA performed the molecular studies, and were involved in acquisition and analysis of data. IP carried out the molecular studies and immunoassays (C2C12 cells). WS and KJ-I carried out the acquisition of data. MAC participated in result analysis and interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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References

1. Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development. 2011;138:3625–37.
2. Tedesco FS, Cossu G. Stem cell therapies for muscle disorders. Curr Opin Neurol. 2012;25:597–603.
3. Ten Broek RW, Grefte S, Von den Hoff JW. Regulatory factors and cell populations involved in skeletal muscle regeneration. J Cell Physiol. 2010;224:7–16.
4. Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature. 1989;337:176–9.
5. Briggs D, Morgan JE. Recent progress in satellite cell/myoblast engraftment – relevance for therapy. FEBS J. 2013;280:4281–93.
6. Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. J Cell Biol. 2002;159:123–34.
7. Asakura A, Rudnicki MA. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol. 2002;30:1339–45.
8. Tanaka KK, Hall JK, Troy AA, Cornelison DD, Majka SM, Olwin BB. Syndecan-4-expressing muscle progenitor cells in the SP engraft as satellite cells during muscle regeneration. Cell Stem Cell. 2009;4:217–25.
9. Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, et al. Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat Commun. 2011;2:499.

10. Sampaolesi M, Torrente Y, Innocenzi A, Tonlorenzi R, D'Antona G, Pellegrino MA, et al. Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. Science. 2003;301:487–92.
11. Sampaolesi M, Blot S, D'Antona G, Granger N, Tonlorenzi R, Innocenzi A, et al. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature. 2006;444:574–9.
12. Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, et al. Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol. 2007;9:255–67.
13. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008;3:301–13.
14. Benchaouir R, Meregalli M, Farini A, D'Antona G, Belicchi M, Goyenvalle A, et al. Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell. 2007;1:646–57.
15. Mitchell KJ, Pannerec A, Cadot B, Parlakian A, Besson V, Gomes ER, et al. Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol. 2010;12:257–66.
16. Greco SJ, Rameshwar P. Mesenchymal stem cells in drug/gene delivery: implications for cell therapy. Ther Deliv. 2012;3:997–1004.
17. De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol. 2003;160:909–18.
18. Meyer S, Yarom R. Muscle regeneration and transplantation enhanced by bone marrow cells. Br J Exp Pathol. 1983;64:15–24.
19. Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S, et al. Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science. 2005;309:314–7.
20. Brzoska E, Grabowska I, Hoser G, Streminska W, Wasilewska D, Machaj EK, et al. Participation of stem cells from human cord blood in skeletal muscle regeneration of SCID mice. Exp Hematol. 2006;34:1262–70.
21. Grabowska I, Streminska W, Janczyk-Ilach K, Machaj EK, Pojda Z, Hoser G, et al. Myogenic potential of mesenchymal stem cells – the case of adhesive fraction of human umbilical cord blood cells. Curr Stem Cell Res Ther. 2013;8:82–90.
22. Grabowska I, Brzoska E, Gawrysiak A, Streminska W, Moraczewski J, Polanski Z, et al. Restricted myogenic potential of mesenchymal stromal cells isolated from umbilical cord. Cell Transplant. 2012;21:1711–26.
23. Corbel SY, Lee A, Yi L, Duenas J, Brazelton TR, Blau HM, et al. Contribution of hematopoietic stem cells to skeletal muscle. Nat Med. 2003;9:1528–32.
24. Chang H, Yoshimoto M, Umeda K, Iwasa T, Mizuno Y, Fukada S, et al. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. FASEB J. 2009;23:1907–19.
25. Darabi R, Gehlbach K, Bachoo RM, Kamath S, Osawa M, Kamm KE, et al. Functional skeletal muscle regeneration from differentiating embryonic stem cells. Nat Med. 2008;14:134–43.
26. Darabi R, Arpke RW, Irion S, Dimos JT, Grskovic M, Kyba M, et al. Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. Cell Stem Cell. 2012;10:610–9.
27. Darabi R, Pan W, Bosnakovski D, Baik J, Kyba M, Perlingeiro RC. Functional myogenic engraftment from mouse iPS cells. Stem Cell Rev. 2011;7:948–57.
28. Nakahata T, Awaya T, Chang H, Mizuno Y, Niwa A, Fukada S, et al. Derivation of engraftable myogenic precursors from murine ES/iPS cells and generation of disease-specific iPS cells from patients with Duchenne muscular dystrophy (DMD) and other diseases. Rinsho Shinkeigaku. 2010;50:889.
29. Skuk D, Goulet M, Roy B, Piette V, Cote CH, Chapdelaine P, et al. First test of a 'high-density injection' protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up. Neuromuscul Disord. 2007;17:38–46.
30. Brzoska E, Kowalewska M, Markowska-Zagrajek A, Kowalski K, Archacka K, Zimowska M, et al. Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. Biol Cell. 2012;104:722–37.
31. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The pleiotropic effects of the SDF-1–CXCR4 axis in organogenesis, regeneration and tumorigenesis. Leukemia. 2006;20:1915–24.
32. Wrobel E, Brzoska E, Moraczewski J. M-cadherin and beta-catenin participate in differentiation of rat satellite cells. Eur J Cell Biol. 2007;86:99–109.
33. Przewozniak M, Czaplicka I, Czerwinska AM, Markowska-Zagrajek A, Moraczewski J, Streminska W, et al. Adhesion proteins – an impact on skeletal myoblast differentiation. PLoS One. 2013;8, e61760.
34. Brzoska E, Grabowska I, Wrobel E, Moraczewski J. Syndecan-4 distribution during the differentiation of satellite cells isolated from soleus muscle treated by phorbol ester and calphostin C. Cell Mol Biol Lett. 2003;8:269–78.
35. Blanco-Bose WE, Yao CC, Kramer RH, Blau HM. Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res. 2001;265:212–20.
36. Lafuste P, Sonnet C, Chazaud B, Dreyfus PA, Gherardi RK, Wewer UM, et al. ADAM12 and alpha9beta1 integrin are instrumental in human myogenic cell differentiation. Mol Biol Cell. 2005;16:861–70.
37. Hadjantonakis AK, Papaioannou VE. Dynamic in vivo imaging and cell tracking using a histone fluorescent protein fusion in mice. BMC Biotechnol. 2004;4:33.
38. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:RESEARCH0034.
39. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. Cell. 2000;102:777–86.
40. Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol. 2006;172:103–13.
41. Maksym RB, Tarnowski M, Grymula K, Tarnowska J, Wysoczynski M, Liu R, et al. The role of stromal-derived factor-1–CXCR7 axis in development and cancer. Eur J Pharmacol. 2009;625:31–40.
42. Archacka K, Denkis A, Brzoska E, Swierczek B, Tarczyluk M, Janczyk-Ilach K, et al. Competence of in vitro cultured mouse embryonic stem cells for myogenic differentiation and fusion with myoblasts. Stem Cells Dev. 2014;23:2455–68.
43. Brzoska E, Bello V, Darribere T, Moraczewski J. Integrin alpha3 subunit participates in myoblast adhesion and fusion in vitro. Differentiation. 2006;74:105–18.
44. Grabowska I, Szeliga A, Moraczewski J, Czaplicka I, Brzoska E. Comparison of satellite cell-derived myoblasts and C2C12 differentiation in two- and three-dimensional cultures: changes in adhesion protein expression. Cell Biol Int. 2011;35:125–33.
45. Powner D, Kopp PM, Monkley SJ, Critchley DR, Berditchevski F. Tetraspanin CD9 in cell migration. Biochem Soc Trans. 2011;39:563–7.
46. Kotha J, Longhurst C, Appling W, Jennings LK. Tetraspanin CD9 regulates beta 1 integrin activation and enhances cell motility to fibronectin via a PI-3 kinase-dependent pathway. Exp Cell Res. 2008;314:1811–22.
47. Castro-Sanchez L, Soto-Guzman A, Navarro-Tito N, Martinez-Orozco R, Salazar EP. Native type IV collagen induces cell migration through a CD9 and DDR1-dependent pathway in MDA-MB-231 breast cancer cells. Eur J Cell Biol. 2010;89:843–52.
48. Charrin S, le Naour F, Silvie O, Milhiet PE, Boucheix C, Rubinstein E. Lateral organization of membrane proteins: tetraspanins spin their web. Biochem J. 2009;420:133–54.
49. Murayama Y, Shinomura Y, Oritani K, Miyagawa J, Yoshida H, Nishida M, et al. The tetraspanin CD9 modulates epidermal growth factor receptor signaling in cancer cells. J Cell Physiol. 2008;216:135–43.
50. Schroder HM, Hoffmann SC, Hecker M, Korff T, Ludwig T. The tetraspanin network modulates MT1-MMP cell surface trafficking. Int J Biochem Cell Biol. 2013;45:1133–44.
51. Hong IK, Kim YM, Jeoung DI, Kim KC, Lee H. Tetraspanin CD9 induces MMP-2 expression by activating p38 MAPK, JNK and c-Jun pathways in human melanoma cells. Exp Mol Med. 2005;37:230–9.
52. Tachibana I, Hemler ME. Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. J Cell Biol. 1999;146:893–904.
53. Schwander M, Leu M, Stumm M, Dorchies OM, Ruegg UT, Schittny J, et al. Beta1 integrins regulate myoblast fusion and sarcomere assembly. Dev Cell. 2003;4:673–85.
54. Charrin S, Latil M, Soave S, Polesskaya A, Chretien F, Boucheix C, et al. Normal muscle regeneration requires tight control of muscle cell fusion by tetraspanins CD9 and CD81. Nat Commun. 2013;4:1674.
55. Xinaris C, Morigi M, Benedetti V, Imberti B, Fabricio AS, Squarcina E, et al. A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. Cell Transplant. 2013;22:423–36.
56. Cencioni C, Capogrossi MC, Napolitano M. The SDF-1/CXCR4 axis in stem cell preconditioning. Cardiovasc Res. 2012;94:400–7.
57. Zemani F, Silvestre JS, Fauvel-Lafeve F, Bruel A, Vilar J, Bieche I, et al. Ex vivo priming of endothelial progenitor cells with SDF-1 before transplantation could increase their proangiogenic potential. Arterioscler Thromb Vasc Biol. 2008;28:644–50.

58. Galvez BG, Sampaolesi M, Brunelli S, Covarello D, Gavina M, Rossi B, et al. Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. J Cell Biol. 2006;174:231–43.
59. Pasha Z, Wang Y, Sheikh R, Zhang D, Zhao T, Ashraf M. Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. Cardiovasc Res. 2008;77:134–42.
60. Franquesa M, Hoogduijn MJ, Baan CC. The impact of mesenchymal stem cell therapy in transplant rejection and tolerance. Curr Opin Organ Transplant. 2012;17:355–61.
61. Hoogduijn MJ, Roemeling-van Rhijn M, Korevaar SS, Engela AU, Weimar W, Baan CC. Immunological aspects of allogeneic and autologous mesenchymal stem cell therapies. Hum Gene Ther. 2011;22:1587–91.
62. Hoogduijn MJ, Dor FJ. Mesenchymal stem cells in transplantation and tissue regeneration. Front Immunol. 2011;2:84.
63. Darabi R, Santos FN, Perlingeiro RC. The therapeutic potential of embryonic and adult stem cells for skeletal muscle regeneration. Stem Cell Rev. 2008;4:217–25.
64. Zheng JK, Wang Y, Karandikar A, Wang Q, Gai H, Liu AL, et al. Skeletal myogenesis by human embryonic stem cells. Cell Res. 2006;16:713–22.
65. Filareto A, Darabi R, Perlingeiro RC. Engraftment of ES-derived myogenic progenitors in a severe mouse model of muscular dystrophy. J Stem Cell Res Ther. 2012;10:S10–001.
66. Barberi T, Bradbury M, Dincer Z, Panagiotakos G, Socci ND, Studer L. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. Nat Med. 2007;13:642–8.
67. Stavropoulos ME, Mengarelli I, Barberi T. Differentiation of multipotent mesenchymal precursors and skeletal myoblasts from human embryonic stem cells. Curr Protoc Stem Cell Biol. 2009;Chapter 1:1F8.
68. Woegerbauer M, Thurnher D, Houben R, Pammer J, Kloimstein P, Heiduschka G, et al. Expression of the tetraspanins CD9, CD37, CD63, and CD151 in Merkel cell carcinoma: strong evidence for a posttranscriptional fine-tuning of CD9 gene expression. Mod Pathol. 2010;23:751–62.
69. Leung KT, Chan KY, Ng PC, Lau TK, Chiu WM, Tsang KS, et al. The tetraspanin CD9 regulates migration, adhesion, and homing of human cord blood CD34+ hematopoietic stem and progenitor cells. Blood. 2011;117:1840–50.

Analiza wewnątrzkomórkowych ścieżek sygnalizacyjnych zaangażowanych w migrację komórek w odpowiedzi na Sdf-1. Wpływ Sdf-1 na zdolność przeszczepianych komórek macierzystych do uczestniczenia w regeneracji mięśnia.

Publikacja oryginalna 3: *Stem cells migration during skeletal muscle regeneration - the role of Sdf-1/Cxcr4 and Sdf-1/Cxcr7 axis*

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Sdf-1 jest chemokiną oddziałującą z dwoma receptorami Cxcr4 oraz Cxcr7. Zdecydowana większość badań dotycząca Sdf-1 opisuje efekty stymulacji receptora Cxcr4, który odpowiada głównie za migrację komórek. Znacznie mniej wiadomo o funkcji receptora Cxcr7. Część z opublikowanych badań świadczy o tym, że pełni jedynie funkcję "gąbki" dla Sdf-1, przez co zmniejsza pobudzenie Cxcr4. Wyniki innych badań pokazują, że jest to funkcjonalny receptor.

W trakcie prowadzonych doświadczeń chciałem sprawdzić, który z receptorów Cxcr4 czy Cxcr7 jest zaangażowany w odpowiedź komórki na Sdf-1. Dodatkowo postanowiłem zidentyfikować wewnątrzkomórkowe ścieżki sygnalizacyjne aktywowane w odpowiedzi na Sdf-1. W tym celu analizowałem hodowane *in vitro* ESC, MSC i mioblasty pierwotne uzyskane z komórek satelitowych izolowanych z mięśni szkieletowych. Komórki te transfekowałem odpowiednio zaprojektowanymi siRNA, co pozwalało mi obniżyć poziom ekspresji Cxcr4 bądź Cxcr7. Następnie komórki traktowałem Sdf-1. Dzięki temu mogłem odróżnić, który receptor jest odpowiedzialny za obserwowaną odpowiedź. Wykazałem, że badane komórki migrują w gradiencie Sdf-1 w sposób zależny wyłącznie od receptora Cxcr4. Sdf-1 nie wpływa na proliferację komórek niezależnie od tego, który receptor był aktywowany. Stymulacja Sdf-1 powoduje wzrost aktywności GTPaz: Rac-1 oraz Cdc42, a więc czynników odpowiedzialnych za rearanżację cytoszkieletu aktynowego uczestniczącego w migracji komórek. Efekt ten zostaje zniesiony po wyciszeniu ekspresji receptora Cxcr4, podczas gdy obniżenie poziomu Cxcr7 nie wpływa na wymienione GTPazy. Wykazałem również, że stymulacja badanych komórek powoduje wzrost poziomu ufosforylowanej (aktywnej) formy FAK (ang. Focal Adhesion Kinase). FAK w ufosforylowanej formie jest odpowiedzialna za tworzenie płytek przylegania, które z kolei są elementem niezbędnym w trakcie migracji komórek. Wyciszenie ekspresji receptora Cxcr4 podobnie jak Cxcr7 powoduje wyraźne obniżenie poziomu ufosforylowanej formy tej kinazy. Wykorzystując technikę mikromacierzy analizowałem również transkryptom ESC stymulowanych Sdf-1, także takich w których wyciszyłem ekspresję Cxcr4 lub Cxcr7. Wyciszenie ekspresji Cxcr4 wpływało na spadek poziomu transkryptów genów zaangażowanych w m.in. na migrację i adhezję. W przypadku wyciszenia ekspresji Cxcr7 spadkowi ulegała przede wszystkim ekspresja genów związanych z białkami cytoszkieletowymi (np. aktyna).

Opisane doświadczenia pozwoliły mi na wykazanie, że Sdf-1 promuje migrację komórek w sposób zależny od receptora Cxcr4 i niezależny od receptora Cxcr7. Aktywacja migracji zachodzi za pośrednictwem GTPaz Cdc42 i Rac1. Wiedząc to, zaplanowałem kolejny etap doświadczeń, w którym komórki stymulowane Sdf-1 i/lub takie, w których wyciszyłem ekspresję receptora Cxcr4 lub Cxcr7, przeszczepiałem do regenerującego mięśnia szkieletowego, Pozwoliło mi to przetestować ich zdolność do uczestniczenia w tworzeniu włókien mięśniowych. Dodatkowo stymulowałem sam mięsień wstrzykując do niego roztwór Sdf-1. Zakładałem, że jeżeli uda mi się zwiększyć zdolność komórek do migracji, to wydajniej będą one uczestniczyć w regeneracji mięśnia. W tych badaniach wykorzystałem mysie ESC oraz mysie mioblasty uzyskane z komórek satelitowych. Wykazałem, że ESC mają bardzo ograniczoną zdolność do zasiedlania mięśnia, jednak stymulacja Sdf-1 powoduje ich wydajniejszą migrację wzdłuż włókien mięśniowych. ESC niestymulowane Sdf-1 oraz te, w których wyciszono ekspresję Cxcr4, formowały agregaty i nie rozprzestrzeniały się w obrębie regenerującego mięśnia. Wyciszenie ekspresji Cxcr7 nie wpływało na zdolność komórek do migracji. Ten wynik potwierdził wcześniejsze obserwacje na temat wpływu Sdf-1 na ESC w hodowli mieszanej z mioblastami. Następnie wykazałem, że mioblasty podane do regenerującego mięśnia są zdolne do współtworzenia włókien mięśniowych. Stymulacja Sdf-1 zwiększa odsetek włókien, w skład których wchodziły badane mioblasty. Ich odróżnienie było możliwe dzięki bakteryjnej β-galaktozydazy obecnej w przeszczepianych mioblastów, które były izolowane z transgenicznej myszy. Podobnie jak w przypadku ESC wyciszenie poziomu ekspresji Cxcr4 powodowało zmniejszenie zdolności do migracji, a w konsekwencji mniejszy odsetek włókien hybrydowych. Cxcr7 nie był zaangażowany w ten proces. Warto jednak zauważyć, że dostarczenie białka Sdf-1 bezpośrednio do mięśnia, skuteczniej indukowało uczestniczenie przeszczepionych komórek w współtworzeniu nowych włókien mięśniowych niż preinkubacja komórek z Sdf-1 przed ich przeszczepieniem do mięśnia. Świadczy to więc o tym, że najistotniejsze jest, aby komórki były wystawione na działanie Sdf-1 w trakcie migracji i fuzji.

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Stem cells migration during skeletal muscle regeneration - the role of Sdf-1/Cxcr4 and Sdf-1/Cxcr7 axis

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ABSTRACT

The skeletal muscle regeneration occurs due to the presence of tissue specific stem cells - satellite cells. These cells, localized between sarcolemma and basal lamina, are bound to muscle fibers and remain quiescent until their activation upon muscle injury. Due to pathological conditions, such as extensive injury or dystrophy, skeletal muscle regeneration is diminished. Among the therapies aiming to ameliorate skeletal muscle diseases are transplantations of the stem cells. In our previous studies we showed that Sdf-1 (stromal derived factor −1) increased migration of stem cells and their fusion with myoblasts *in vitro*. Importantly, we identified that Sdf-1 caused an increase in the expression of tetraspanin CD9 - adhesion protein involved in myoblasts fusion. In the current study we aimed to uncover the details of molecular mechanism of Sdf-1 action. We focused at the Sdf-1 receptors - Cxcr4 and Cxcr7, as well as signaling pathways induced by these molecules in primary myoblasts, as well as various stem cells - mesenchymal stem cells and embryonic stem cells, i.e. the cells of different migration and myogenic potential. We showed that Sdf-1 altered actin organization *via* FAK (focal adhesion kinase), Cdc42 (cell division control protein 42), and Rac-1 (Ras-Related C3 Botulinum Toxin Substrate 1). Moreover, we showed that Sdf-1 modified the transcription profile of genes encoding factors engaged in cells adhesion and migration. As the result, cells such as primary myoblasts or embryonic stem cells, became characterized by more effective migration when transplanted into regenerating muscle.

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Introduction

The skeletal muscle regeneration occurs due to the presence of stem cells called satellite cells (SCs) that are localized between sarcolemma and basal lamina. The role of SCs in skeletal muscle repair is unquestionable (reviewed in ref. 1). Unfortunately, due to aging, extensive damages or various pathological states, for example muscular dystrophy, muscle reconstruction is diminished.[2-4] Stem cells transplantation belongs to the therapeutic approaches aiming to improve muscle regeneration (reviewed in ref. 5). In the initial studies, focusing on the skeletal muscle cell therapies, SCs and primary myoblasts, due to their natural function, were the first choice of cells tested (reviewed in refs. 6, 7). Under physiological conditions, after muscle injury, SCs become activated what leads to the cell cycle re-entry, proliferation, and finally their differentiation into myoblasts that fuse and reconstruct myotubes and then muscle fibers. In the 80s of XX century Partridge and collaborators documented that wild-type myoblasts injected to muscle of dystrophic mice (*mdx* mice) were able to reconstruct muscle fibers and restore the dystrophin synthesis.[8] As demonstrated later, the improvement in the skeletal muscle regeneration was observed after transplantation of undifferentiated, purified satellite cells population, rather than satellite cells derived myoblasts.[9,10] In the 90s many clinical trials based on the model described by Partridge were conducted, however, the results were not satisfactory (reviewed in refs. 6, 11). Transplanted cells were able to participate in the muscle regeneration and partially restore dystrophin expression but no functional long-term improvement was observed.[12-14]

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The most important obstacles in myoblast transplantation include specific immune response against transplanted cells, limited migration within the muscle, and massive apoptosis of transplanted cells (reviewed in refs. 7, 15, 16). The limited migration ability of transplanted myoblasts was shown in many studies.[17-20] Thus, many lines of evidence documented that injected myoblasts accumulate within the site of injection and only few reports showed that they could migrate up to 1 cm in depth from the monkey (*Macaca mulata*) muscle surface.[21] Importantly, co-injected growth factors such as bFGF (basic fibroblast growth factor) and IGF-1 (insulin like growth factor), improved migration of monkey (*Macaca mulata*) myoblasts transplanted into *biceps brachii*. However, myofibers formed with the participation of these cells were detectable only near the injection site. Moreover, analyzed myoblasts were not able to fuse with undamaged muscle fibers, regardless of the growth factors used.[22] In our own studies we showed that the Sdf-1 could improve migration of satellite cell derived myoblasts and C2C12 myoblasts *in vitro* in metalloproteinase (MMP) dependent manner.[23] We also documented that Sdf-1 treatment enhanced embryonic stem cells (ESCs) and bone marrow derived mesenchymal stem cells (BM-MSC) migration and fusion with myoblasts *in vitro*, what was connected with the increase in tetraspanin CD9 expression.[24]

In the current study we investigated which molecular pathways induced by Sdf-1 lead to the increased migration. We hypothesized that stimulation of transplanted cells migration using Sdf-1 improves their ability to participate in muscle repair. To verify this hypothesis we analyzed various stem cell populations - mouse primary myoblasts derived from SCs, human mesenchymal stem cells isolated from umbilical cord connective tissue, i.e., Wharton's jelly (WJ-MSCs), and mouse embryonic stem cells (ESCs). Our choice based on the previous studies in that we documented that these stem cells are able to undergo myogenic differentiation and also to participate in the skeletal muscle regeneration.[24-26]

Multipotent mesenchymal stem cells (MSCs) could be derived from different sources, such as bone marrow, adipose tissue, Wharton jelly (umbilical cord connective tissue), umbilical cord blood, skin, dental pulp, spleen, lung, and also skeletal muscles (reviewed in refs. 27, 28). Various populations of mesenchymal stem cells were able to improve skeletal muscle reconstruction.[29-31] Myogenic differentiation of the pluripotent stem cells, such as embryonic stem cells (ESCs), which are characterized by unlimited potential to proliferate and ability to differentiate into any given tissue, has been also documented (reviewed in refs. 32, 33). An efficient protocol allowing derivation of myoblasts from ESCs, based on the supplementation of culture medium with factors inducing mesoderm formation and myogenic differentiation, was proposed only recently and obtained myoblasts were tested both *in vitro* and *in vivo*.[34] Cells derived from ESCs when transplanted into tibialis anterior muscles of *mdx* mice were able to form muscle fibers and also to differentiate into Pax7-expressing cells that resembled SCs.[34] However, methods improving homing of these cells to the site of the injury via improvement of their migration are still not readily available.

In the current study we compared the reaction of primary myoblasts, WJ-MSC, as well as ESCs to Sdf-1 treatment, which—as we previously shown—is a potent factor improving skeletal muscle regeneration.[23,24] First we analyzed changes in transcription profile and the signaling pathways engaged in stem cells response to Sdf-1 treatment. Next, we concentrated on the role of Sdf-1 receptors i.e. CXCR7 and CXCR4 in stem cells migration both *in vitro* and *in vivo*. Then, we examined if Sdf-1 pretreatment of stem cells with Sdf-1 or co-injection of these cytokine could improve participation of tested cells in the skeletal muscle regeneration.

Materials and methods

All the experiments were performed with the approval of Local Ethical Commission No 1 in Warsaw – permission no 240/2012.

Cells cultures

Satellite cells – derived myoblasts (primary myoblasts)

Satellite cells were isolated from the gastrocnemius muscles of 3 months old C57Bl6N male mice carrying the lacZ transgene in the ROSA26 locus. Mice were sacrificed by cervical dislocation. Muscle fibers were isolated according to previously described protocol.[35] Briefly, muscles were dissected and digested with 0.2% collagenase type I (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) at 37°C in 5% CO_2 for 60 min. Next, single muscle fibers were transferred to DMEM containing 10% horse serum (HS, Life Technologies) and 1% penicillin/streptomycin antibiotics (AB, Life Technologies). Suspension of muscle fibers was passed through a syringe needle (21G) and cleared by filtration through 40 μm cell strainer. Obtained satellite cells were plated in 6-well culture dishes coated with Matrigel Matrix Growth Factor Reduced (BD Biosciences). Primary myoblasts were maintained in so called "growth medium," i.e., DMEM supplemented with 20% fetal bovine serum (FBS), 10% HS, 0.5% chicken embryo extract (CEE, Life Technologies) and 1% AB.

Mesenchymal stem cells derived from Wharton jelly (WJ-MSCs)

WJ-MSCs were kindly provided by prof. Zygmunt Pojda (Department of Molecular and Translational Oncology,

Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland). WJ-MSCs were seeded and cultured in DMEM (Life Technologies) supplemented with 15% heat inactivated FBS (hiFBS) and 1% AB.

Mouse embryonic stem cells (ESCs)

ESCs constitutively expressing histone H2B-GFP were provided by Dr. Kat Hadjantonakis.[36] Mitomycin-inactivated mouse embryonic fibroblasts (MEFs), that served as feeder layer for ES cells, were plated on 1% gelatin coated culture dishes (Sigma-Aldrich) and cultured in DMEM supplemented with 10% FBS and 1% AB. Twenty four hours later ESCs were seeded onto the inactivated MEFs and cultured in knockout DMEM (Life Technologies) supplemented with 10% serum replacement (SR, Life Technologies), 0.1 mM nonessential amino acids (Sigma-Aldrich), 2 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1% AB, and 500 U/ml leukemia inhibitory factor (LIF, Chemicon). Prior to transfection with siRNA, ESCs were separated from MEFs by pre-plating and cultured in cultured dishes coated with 10% Matrigel Matrix Growth Factor Reduced (BD Biosciences) in DMEM until the time of further manipulations.

Morphological analyses

The morphology of primary myoblasts, WJ-MSCs, and ESCs was analyzed using Nikon Eclipse TE200 microscope equipped with Hoffman contrast.

Cells transfection

Primary myoblasts, WJ-MSCs, and ESCs were plated into culture dishes and after reaching 50-60% of confluency transfected with Silencer Select Pre-designed siRNA (Life Technologies) complementary to mRNAs encoding either *Cxcr4* (ID:s64091) or *Cxcr7* (ID:s64124). Appropriate negative control siRNA was used according to manufacturer's recomendation. siRNA duplexes were diluted in DMEM to 100 pmol concentration and Lipofectamine RNAiMAX (Life Technologies) was added according to manufacturer's instructions. The Sdf-1 (10 ng/μl) was added 24 h after transfection. The cells were collected 48 h post-Sdf-1 treatment and processed either for mRNA isolation, followed by qRT-PCR, immunolocalization, Western blotting, G-LISA or for transplantation into injured and regenerating gastrocniemus muscles. The efficiency of CXCR4 or CXCR7 down regulation was assessed by qRT-PCR and Western-blot.

Quantified real time PCR (qRT-PCR)

Total RNA was isolated from primary myoblasts, WJ-MSCs, and ESCs using mirVana Isolation Kit (Life Technlogies), according to the manufacturer's protocol. RNA was extracted from biological triplicates (3 independent cell cultures per each experiment). 250 ng of RNA from each sample was reverse-transcribed using the SuperScript II Reverse Transcriptase (Life Technologies) according to the manufacturer's protocol. Next, mRNA levels were examined using Quantitative real-time PCR analysis (qPCR) with TaqMan assays (Life Technologies) for the following genes: *CXCR4* [Mm01996749], *CXCR7* [Mm02619632], *Rac-1* [Mm01331626], *Cdc42* [Mm01194005], focal adhesion kinase (*FAK*) [Mm00552827], and actin [Mm01268569]. Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) [Mm01545399] was used as the reference gene. All reactions were performed in triplicates. qPCR was performed with the TaqMan Gene Expression Master Mix (Life Technologies) using LightCycler 480 (Roche Applied Sciences) according to manufacturer's instruction. The conditions of RT-qPCR were as follows: reverse transcription: 25°C for 10 min, 42°C for 60 min, 85°C for 5 min, qPCR: 50°C for 2 min, template denaturation 95°C for 10 min, 45 cycles of 95°C for 15 sec and 60°C for 60 sec. Threshold-cycle (Ct) values of the analyzed amplicons were determined with LightCycler® 480 Software (Roche Applied Science). Expression levels were calculated with $2^{-(\Delta CT)}$ formula using relative quantification tool in LightCycler® 480 Software. Expression levels and standard deviations for each gene was visualized as the column charts using GraphPad Software (La Jolla, CA, USA). Reference gene *Hprt1* displayed high expression stability. Results were analyzed using using GraphPad Software and non-paired t-test was performed to compare treated with the control cells. The differences were considered statistically significant when $p < 0.05$ (marked with asterisks).

Cell proliferation assay

Primary myoblasts, WJ-MSCs, and ESCs were incubated in 0.5 μM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) in PBS at 37°C for 10 min. Cells were rinsed in PBS and cultured for 2 days in the culture medium appropriate for each cell type, under standard conditions. Next, cells were rinsed in PBS and subjected to flow cytometry analysis (BD FACSCALIBUR, BD Biosciences) using CellQuestPro software. Unlabeled cells (negative control) and cells analyzed directly after labeling with CFSE (positive control) were included into each experiment. Three independent experiments were performed. Results were analyzed using GraphPad Software and non-paired t-test was performed to compare treated with the control cells. The differences were considered statistically significant when $p < 0.05$ (marked with asterisks).

Migration assay

Migration of myoblasts, WJ-MSCs, and ESCs was analyzed using scratch wound healing assay.[37] Briefly, cells were plated in the culture dish and cultured until they reached 90% of confluency. Next, the cells were scratched from the plate using plastic tip to create the "wound." The wound healing manifested by the ability of the cells to refill the created gap was monitored after 48h of culture. Three independent experiments were performed. Results were analyzed using using GraphPad Software and non-paired t-test was performed to compare treated with the control cells. The differences were considered statistically significant when $p < 0.05$ (marked on charts with asterisks).

Analysis of Rac-1 and Cdc42 activity

Primary myoblasts, ESCs and WJ-MSCs were cultured as described above. Thirty min after Sdf-1 treatment cells were lysed in culture dishes, lysates collected and frozen in liquid nitrogen. Active Cdc42 and Rac-1 were analyzed using the G-LISA activation assay kit (Cytoskeleton, Inc.) according to the manufacturer's instructions. The chemiluminescence signal was detected using the μQuant (Biotek Instruments) microplate reader. Three independent experiments were performed. Results were analyzed using GraphPad Software and non-paired t-test was performed to compare treated with the control cells. The differences were considered statistically significant when $p < 0.05$ (marked on charts with asterisks).

Microarray analysis

ESCs were cultured and either treated with Sdf-1 or transfected with Silencer Select Pre-designed siRNA (Life Technologies) complementary to mRNAs encoding either *CXCR4* (ID:s64091) or *CXCR7* (ID:s64124) as described above. Total RNA was isolated using mirVana Isolation Kit (Life Technlogies). Next, its integrity was checked with 2100 Bioanalyzer (Agilent Technologies) using RNA 6000 NAno LAb Chip kit (Agilent Technologies). All RNA samples had integrity number above 8.5. 100 ng of total RNA for each sample was biotin labeled with the TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip® (Epicentre Biotechnologies). Labeled RNA was purified with RNeasy® MinElute® Cleanup Kit (Qiagen) and hybridized onto MouseRef-8 v2.0 Expression BeadChip (Illumina Inc.) according to manufacturer's instructions. Arrays were scanned with an HiScan®SQ System (Illumina Inc.). Raw data were imported to GenomeStudio (Illumina) and the average signal intensities were analyzed in Partek Genomic Suite (Partek, Inc.) v. 6.6 after quantile normalization and Log2 transformation. Qualitative analysis was performed, e.g. Principal Component Analysis, in order to identify outliers and artifacts on the microarray. After quality check the 2-way ANOVA (Analysis of Variance) model by using Method of Moments[38] was performed on the data and lists of significantly and differentially expressed genes between biological variants (with the cutoff values: p-value < 0.05, $-1.3 >$ Fold Change > 1.3) were created. Fisher's Least Significant Difference (LSD) was used as the contrast method[39] to compare: ES-Cxcr4 (ESCs transfected with siRNA complementary to CXCX4 mRNA) vs ES-Sdf-1 (ESCs treated with Sdf-1) and ES-Cxcr7 (ESCs transfected with siRNA complementary to CXCX7 mRNA) vs ES-Sdf-1. Unsupervised hierarchical clustering was performed on the selected lists to in order to find genes and samples with similar profiles. Gene networks were created by interposing the results onto the database of Ingenuity containing information about gene functions with the use if Ingenuity Pathway Analysis tool.

Muscle injury and cells transplantation

To induce regeneration of skeletal muscles, 3-month old male BALB/c mice were anesthetized and their gastrocniemus muscles were injected with 50 μl of cardiotoxin (CTX) from Naja mossambica (10 μM in PBS, Sigma-Aldrich). After the procedure mice were kept under standard conditions with free access to food and water. Twenty four hours later control cells, Sdf-1 treated cells, or cells in that expression of *Cxcr4* or *Cxcr7* was silenced were injected into injured muscles. The number of transplanted cells varied, i.e. 0.5 million of myoblasts, 0.2 million of WJ-MSCs or 1 million of ESCs, suspended in 50 μl of PBS, was transplanted. Moreover, regenerating gastrocnemius muscle was injected with Sdf-1 (100 ng per 20 μl of 0.9% NaCl) or 20 μl of 0.9% NaCl (saline treated muscles served controls). Sdf-1 and NaCl was injected at the opposite ends of the muscles than the transplanted cells. After 7 or 14 d after injury, i.e., days of regeneration, muscles were dissected and analyzed (immunocytochemistry and histochemistry). Localization of transplanted cells within the muscle was based on the expression of appropriate markers. Satellite cells, from which primary myoblasts were derived, were isolated from 3-month old C57Bl6N male mice carrying the lacZ transgene in the ROSA26 locus. WJ-MSCs were localized on the basis of human nuclear antigen. ESCs were localized on the basis of the expression of H2B-GFP.

Immunocytochemistry

Selected antigens were immunolocalized in *in vitro* cultured cells, isolated at day 7 of regeneration muscle fibers, as well as in muscle sections (cross and longitudinal). Cells or isolated muscle fibers were fixed with 3% PFA for 10 min, washed with PBS and stored in 4°C. Muscles were dissected

7 or 14 d after injury and cells transplantation. They were frozen in isopentane cooled with liquid nitrogen, transferred into −80°C, and cut into 7 μm-thick sections with cryomicrotome (Microm HM505N) and stored in 4°C. Cryosections were hydrated in PBS, fixed in 3% PFA, and washed with PBS. To obtain longitudinal sections dissected muscles were fixed with Bouin's solution, dehydrated and embed into paraffin blocks. Paraffin blocks were cut for 9 μm-thick slices and placed on covered with 0.5% gelatin in water glass slides and then dried in 40°C. Paraffin sections were stored in 4°C and rehydrated before immunolocalization.

Next, cells or muscle sections were permeabilized with 0.1% Triton X-100/PBS (Sigma-Aldrich), and incubated with 0.25% glycine (Sigma-Aldrich). Non-specific binding of antibodies was blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) supplemented with 2% donkey serum (Sigma-Aldrich) in PBS, at room temperature, for 1h. Next, samples were incubated for 2 h with primary antibodies diluted 1:100 in 3% BSA in PBS, overnight, washed with PBS, and incubated at room temperature with secondary antibodies diluted 1: 200 in 1.5 % BSA in PBS. After washing with PBS, cell nuclei were visualized by incubation with DraQ5 (Biostatus Limited) diluted 1:1000 in PBS for 10 min. Specimens were mounted with Fluorescent Mounting Medium (Dako Cytomation). After the procedure was completed samples were analyzed using confocal microscope Axiovert 100M (Zeiss) and LSM 510 software. The following primary antibodies were used: chicken polyclonal anti-β-galactosidase (Abcam), mouse monoclonal anti-human nuclear antigen (Abcam), mouse monoclonal anti-Green Fluorescent Protein (GFP) (Abcam), rabbit polyclonal anti-Myod1 (Abcam), mouse monoclonal anti-Cdc42 (SantaCruz Biotechnology), rabbit polyclonal anti-Rac-1 (SantaCruz Biotechnology), rabbit polyclonal anti-FAK (SantaCruz Biotechnology), and rabbit polyclonal anti-laminin (Sigma-Aldrich). The following secondary antibodies were used: anti-mouse igG Alexa Fluor 488, anti-rabbit igG Alexa Fluor 566, anti-rabbit igG Alexa Fluor 488, and anti-chicken igG Alexa Fluor 488. All secondary antibodies were purchased from Life Technologies. Actin cytoskeleton was localized using falloidin conjugated with TRITC (Sigma). Three independent experiments were performed for each analysis.

Western blotting

Proteins were isolated from cells or gastrocnemius muscles using cOmplete Lysis-M EDTA-free kit (Roche Applied Science). Twenty-five μg of total protein lysate were denatured by boiling in Laemmli buffer, separated using SDS-Page electrophoresis, and transferred to PVDF membranes (Roche Applied Science). The membranes were blocked with 5% Blotto (BioRad)/TBS for 1h and incubated with primary antibodies diluted 1:2000 in 5% Blotto (BioRad)/TBS, at 4°C, overnight, followed by secondary antibodies diluted 1:20000, at room temperature, for 2 h. Next, protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to chemiluminescence positive film (Amersham Hyperfilm ECL, GE Healthcare). The obtained results were analyzed with GelDoc2000 using Quantity One software (BioRad). The density of examined bands was compared to density of tubulin bands. The following primary antibodies were used: rabbit polyclonal anti-Cxcr4 (Abcam), rabbit polyclonal anti-Cxcr7 (Abcam), rabbit polyclonal anti-pFAK (Cell Signaling), rabbit polyclonal anti-FAK (SantaCruz Biotechnology), mouse monoclonal anti-Cdc42 (SantaCruz Biotechnology), rabbit polyclonal anti-Rac-1 (SantaCruz Biotechnology), mouse monoclonal anti-actin (Abcam), and mouse monoclonal anti-tubulin (Sigma-Aldrich). Secondary antibodies used were: peroxidase-conjugate rabbit anti-mouse igg (Sigma-Aldrich) and peroxidase-conjugate goat anti-rabbit igg (Sigma-Aldrich). Three independent experiments were performed.

Flow cytometry analysis

Gastrocnemius muscles that received ESCs constitutively expressing histone H2B-GFP were isolated at day 7 and 14 of regeneration. Next, they were digested with 0.15% pronase (Sigma Aldrich) in Ham's F12 medium (Life Technologies) buffered with 10 mM HEPES (Life TEchnolofies), containing 10% fetal calf serum (FCS), at 37°C, for 1.5 h. Obtained cell suspension was filtered through 40 μm cell strainer. Then, cells were fixed in a 3% PFA in PBS, washed with PBS, and analyzed with FACSCalibur (Becton-Dickinson) equipped with a 488-nm argon laser to detect GFP signal. The cells were also incubated with rabbit polyclonal anti-Myf5 antibody (Abcam) diluted in 3% BSA in PBS 1:100, at room temperature, for 1 h, followed by secondary antibody anti-rabbit igG Alexa Fluor 566. Three data parameters were acquired and stored: FSC, SSC and fluorescence 1 – FL1 (fluorescein isothiocyanate, FITC). CellQuest application, version 1.2, was used for the analysis. Three independent experiments were performed. Results were analyzed GraphPad Software and non-paired t-test was performed to compare treated with the control cells. The differences were considered statistically significant when $p < 0.05$ (marked on charts with asterisks).

Results

Sdf-1 induces stem cells migration but not proliferation

We analyzed primary myoblasts, WJ-MSCs, or ESCs which were treated with Sdf-1 alone or transfected with siRNA

complementary to mRNAs encoding either Cxcr4 or Cxcr7 and treated with Sdf-1, along with control, i.e. untreated cells. The Cxcr4 or Cxcr7 silencing assessed at mRNA level was proved to be efficient. siRNA complementary to Cxcr4 mRNA decreased the level of this transcript to 28% +/− 8% in primary myoblasts, 49% +/− 16% in WJ-MSCs, and to 34% +/− 8% in ESCs, as compared to control, i.e., cells of each type that where neither treated with Sdf-1 nor transfected with siRNAs (Fig. 1A). siRNA complementary to mRNA encoding Cxcr7 decreased the level of Cxcr7 transcripts to 34% +/− 11% in primary myoblasts, 38% +/− 18% in WJ-MSCs and 41 % +/− 13% in ESCs, as compared to control (Fig. 1A). Sdf-1 treatment did not significantly change the level of Cxcr4 or Cxcr7 mRNA (Fig. 1A). The changes in CXCR4 and CXCR7 level in treated cells were also pronounced at protein level (Fig. 1B).

Scratch migration assay revealed that in the response to Sdf-1 gradient primary myoblasts, WJ-MSC and ESCs migrate more effectively (Fig. 2A). Migration of all types of examined cells depended on Cxcr4 receptor - silencing of its expression decreased this process (Fig. 2A). Cxcr7 silencing did not significantly impact at the cell migration in performed assay. Next, we tested whether Sdf-1 controls the ability of primary myoblasts, WJ-MSCs, and ESCs to proliferate. CFSE test allowed us to estimate the proportion of cells that did not divide, divided once or more than twice. It proved that Sdf-1 treatment did not change myoblasts, WJ-MSCs, as well as ESCs proliferation rate. Neither Cxcr4 nor Cxcr7 expression silencing affected primary myoblasts and WJ-MSCs divisions (Fig. 2B). Interestingly, Cxcr7 silencing significantly increased ESCs proliferation (Fig. 2B).

The signaling pathways in actin organization in stem cells after Sdf-1 treatment

Next, we analyzed the changes in actin cytoskeleton organization and which signaling pathways were involved in the activation of the analyzed cells migration in the response to Sdf-1. We chose to analyze Cdc42 (cell division control protein 42), Rac-1 (Ras-Related C3 Botulinum Toxin Substrate 1), and focal adhesion kinase (FAK), i.e. the factors known to participate in the processes associated with cell migration such as actin polymerization and focal contacts formation.[40-42] After Sdf-1 stimulation the morphology of the cells and organization of actin cytoskeleton changed, i.e., all analyzed cells formed numerous stress fibers and filopodia (Fig. 3A). In Sdf-1 treated cells the actin filaments were more abundant (Fig. 3A). This effect was reversed by Cxcr4 silencing, what correlated with the decrease in actin expression at mRNA and protein levels (Fig. 3B and C). The SCs and WJ-MSCs in that Cxcr4 expression was silenced were

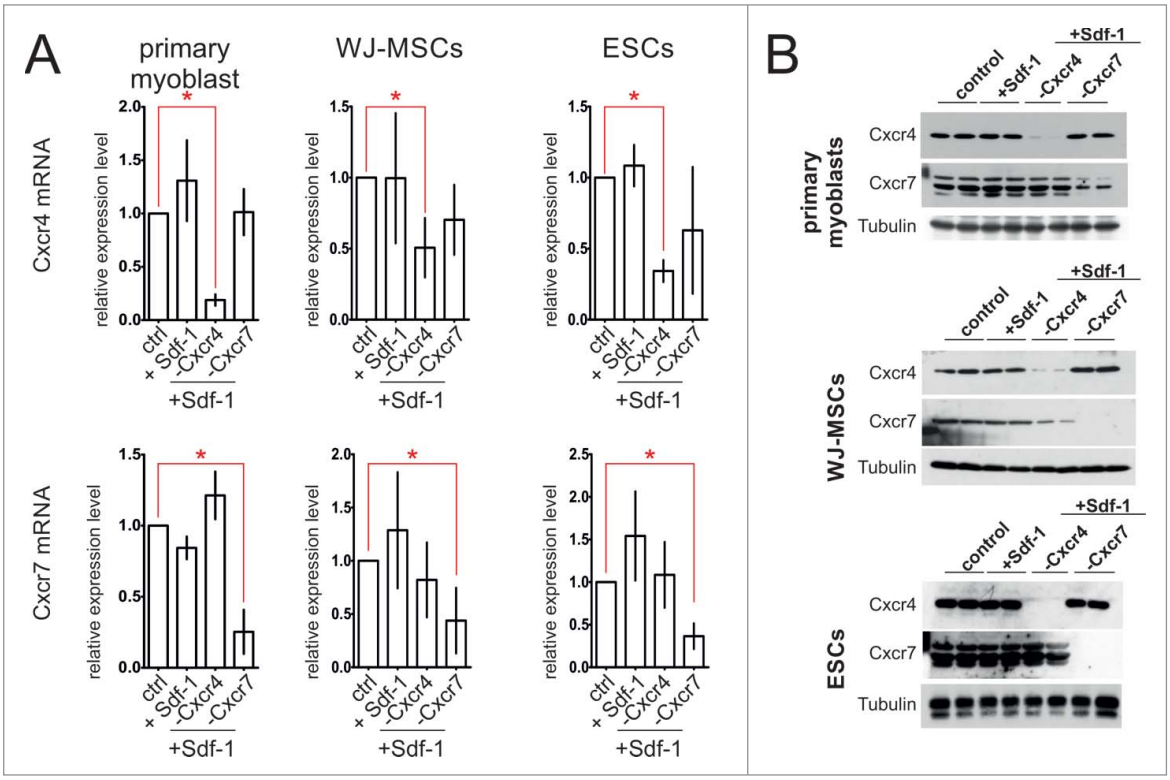

Figure 1. The Cxcr4 and Cxcr7 level in *in vitro* cultured primary myoblasts, WJ-MSCs, and ESCs 48h after transfection with siRNA and Sdf-1 treatment. (A) The level of mRNA encoding Cxcr4 and Cxcr7. Obtained data is presented as mean ± standard deviation. Student's non-paired t-test was used for statistical analyses. Asterisk marks significant differences ($P < 0.05$). (B) Western blotting of Cxcr4, Cxcr7, and tubulin.

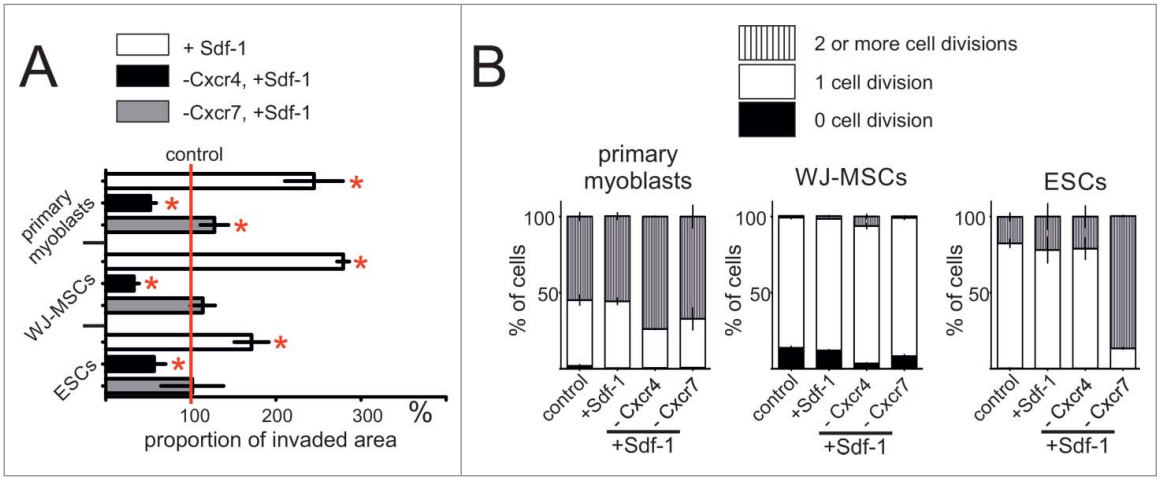

Figure 2. Sdf-1 impact at *in vitro* cultured primary myoblasts, WJ-MSCs, and ESCs migration and proliferation. (A) Proportion of invaded area calculated from the results of scratch wound assay. Analysis was performed at day second after the scratch wound formation. (B) Results of CFSE test documenting the proliferation rate of studied cells. Analysis was performed after 2 d of culture subsequently CFSE staining. Obtained data is presented as mean ± standard deviation. Student's non-paired t-test was used for statistical analyses. Asterisk marks significant differences ($P < 0.05$).

characterized by changes in Cdc42, Rac-1 and FAK localization (Fig. 3D and E).

However, Sdf-1 did not impact at the levels of Cdc42, Rac-1, and FAK proteins in primary myoblasts, WJ-MSCs, or ESCs (Fig. 4A). Silencing of Cxcr4 or Cxcr7 expression slightly decreased the level of Cdc42 protein (Fig. 4A). Importantly, Sdf-1 caused the significant changes in the activity of studied proteins (Fig. 4A and B). The activity of Cdc42 and Rac-1 GTPases was higher in Sdf-1 treated myoblasts, WJ-MSCs, and also ESCs, as compared to the untreated cells (Fig. 4B). Silencing of Cxcr4 but not Cxcr7 abolished the impact of Sdf-1 at the activity of Cdc42 and Rac-1 GTPases. As far as active, phosphorylated form of FAK (pFAK), is concerned it was detectable in control myoblasts, WJ-MSCs, and ESCs (Fig. 4A). Sdf-1 caused increase in the level of pFAK in myoblasts and WJ-MSCs, but not in ESCs. However, in all studied cell types the effect of Sdf-1 on FAK phosphorylation was lost when expression of both its receptors, i.e. Cxcr4 and Cxcr7, was silenced (Fig. 4A).

Changes in transcription profile in ESCs after Sdf-1 treatment

To analyze the changes in the transcriptome provoked by Sdf-1 we decided to use ESCs because in these cells the changes in morphology and cytoskeleton organization was the best pronounced after Sdf-1 treatment. mRNA isolated from control ESCs, as well as cells that were Sdf-1 treated, Sdf-1 treated and transfected with siRNA complementary to mRNAs encoding either Cxcr4 or Cxcr7, was analyzed using microarray technique (Fig. 5). Analysis of variance (ANOVA) allowed to create lists of genes significantly changed in ESCs that were Sdf-1 treated and transfected with appropriate siRNA, as compared to cells treated only with Sdf-1 (with the cutoff values: p-value< 0.05, −1.3>Fold Change>1.3). This analysis revealed that Sdf-1, acting via Cxcr4 receptor, regulates the expression of 90 transcripts, while acting via Cxcr7 receptor affects the expression of 113 transcripts (Fig. S1). Using Ingenuity Pathway Analysis we showed that Sdf-1 impacts at the expression of many genes encoding proteins engaged in cells adhesion and migration (Fig. 5), including transcripts encoding proteins engaged directly or indirectly in actin and adhesion proteins expression. Sdf-1 acting via Cxcr4, but not Cxcr7, regulates the expression of mRNA encoding adhesion proteins such as tetraspanin CD9 and ADAM9 (a disintegrin and metalloproteinase 9). Sdf-1 acting via Cxcr4 influences the expression of transcripts encoding cytoskeleton proteins present in skeletal muscle fibers, such as actin or α actin (ACTA1). Activation of this signaling pathway also induced the expression of calpain small subunit 1 (CAPNS1) that belongs to the family of calcium-dependent, non-lysosomal cysteine proteases. Sdf-1 acting via Cxcr7 impacted the levels of mRNAs encoding F-actin and ACTA1. It also reduced the expression of mRNAs encoding calpains, such as calpain 5 (CAPN 5) and CAPNS1. Summarizing, transcriptome analysis confirmed important role of Sdf-1 in the activation of migration and allowed us to pinpoint and distinguish the targets of pathways activated by Sdf-1 binding to Cxcr4 or Cxcr7.

Regeneration of injured skeletal muscles treated with Sdf-1 and stem cells

Next, we decided to analyze if Sdf-1 pretreatment of transplanted cells or Sdf-1 co-injection with transplanted cells

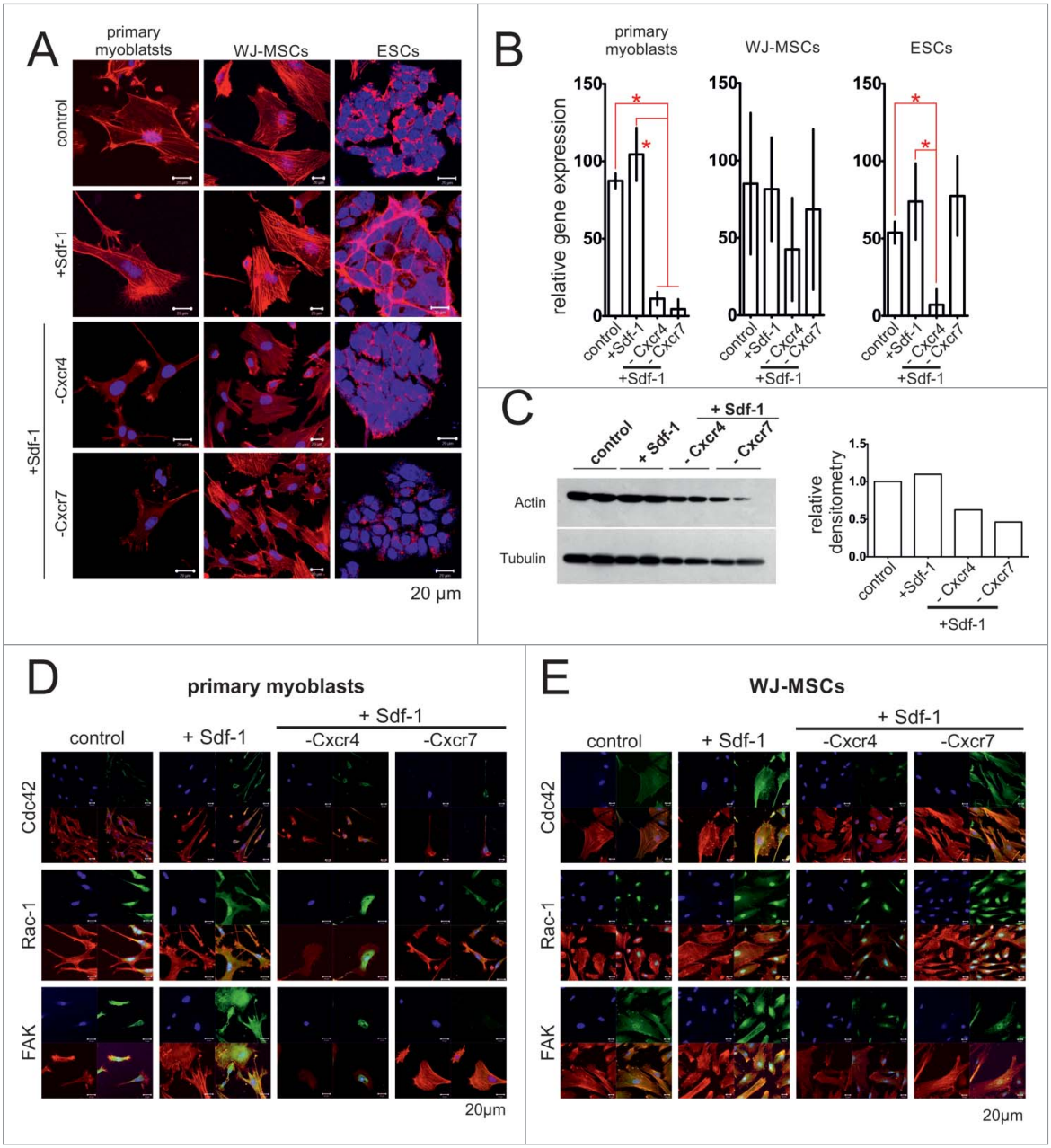

Figure 3. Sdf-1 impact at actin, FAK, Rac-1 and Cdc42 in *in vitro* cultured primary myoblasts, WJ-MSCs, and ESCs. (A) Immunolocalization of actin (red - actin, blue - chromatin). (B) The level of mRNA encoding actin. Obtained data is presented as mean ± standard deviation. Student's non-paired t-test was used for statistical analyses. Asterisk marks significant differences ($P < 0.05$). (C) Western blotting of actin in ESCs. (C) Localization of FAK, Rac-1 and Cdc42 in primary myoblasts (blue – chromatin, red – immunolocalization of actin, green – immunolocalization of studied proteins). (D) Localization of FAK, Rac-1 and Cdc42 in WJ-MSCs (blue – chromatin, red – immunolocalization of actin, green – immunolocalization of studied proteins).

could improve participation of stem cells in muscle regeneration. Again we also focused at the role of Cxcr4 and Cxcr7 in the migration of tested cells in regenerating muscle. Control, i.e., untreated primary myoblasts, WJ-MSCs, or ESCs, as wells as cells treated with Sdf-1 alone or treated with Sdf-1 and transfected with siRNA complementary to mRNAs encoding either Cxcr4 or Cxcr7 were transplanted to control muscles injected with 0.9% NaCl (saline) or muscles injected with Sdf-1 in 0.9% NaCl (Fig. 6). One muscle of each muscle pair was injected with saline, while another, i.e. contralateral, with Sdf-1. Importantly, Sdf-1 was injected at the opposite end of the muscle in the relation to the site of cells transplantation (approximately 1 cm distance). Sdf-1 was co-injected with transplanted cells.

First, we focused at the morphology of all groups of treated muscles, followed the localization of transplanted cells, as well as, the efficiency of their migration and muscle homing (Fig. 7). Primary myoblast were identified as

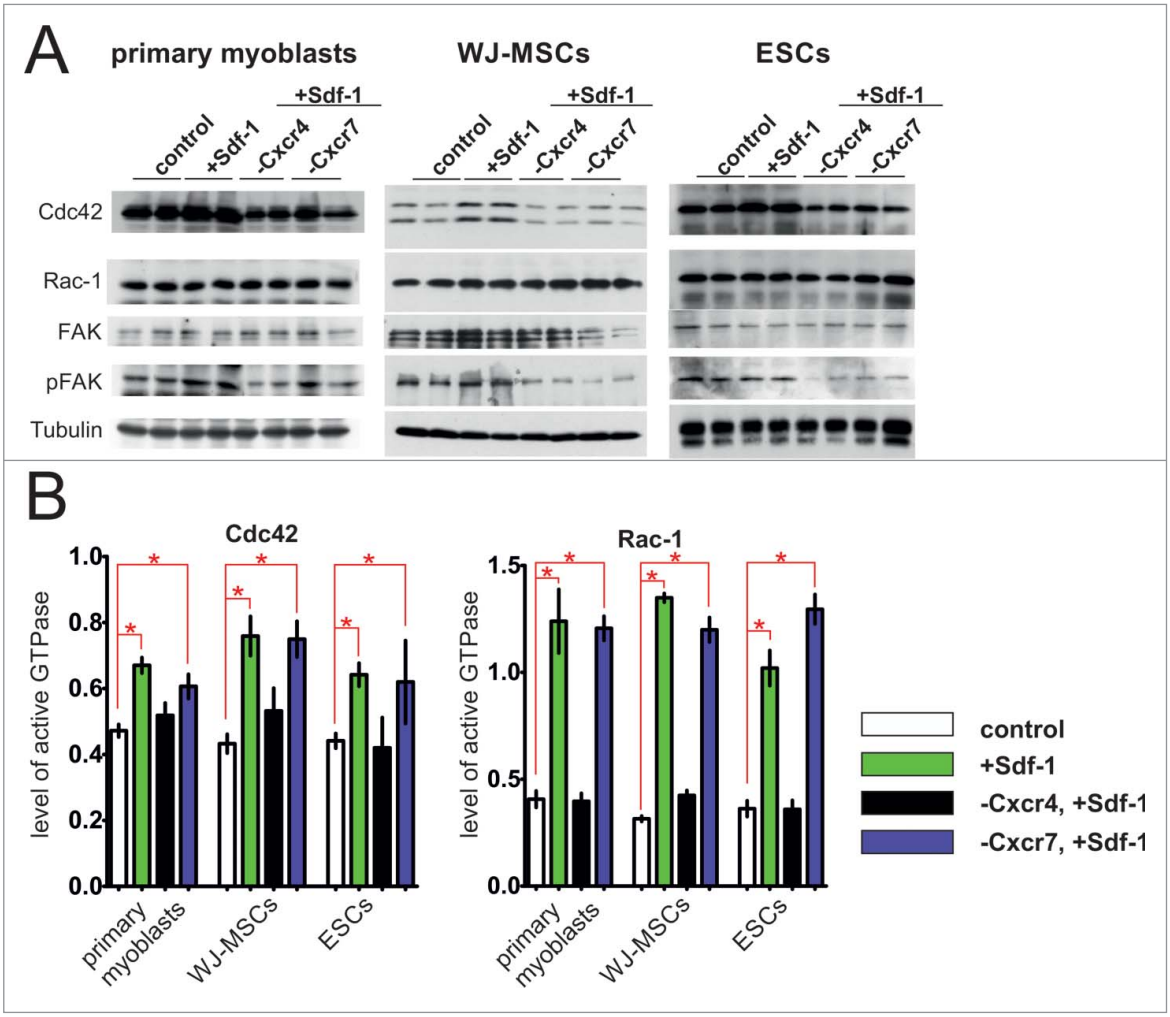

Figure 4. Sdf-1 impact at FAK, Rac-1 and Cdc42 level and activation *in vitro* cultured primary myoblasts, WJ-MSCs, and ESCs. (A) Western blotting of Cxcr4, Cxcr7, FAK, phosphorylated FAK (pFAK), Rac-1, Cdc42, and tubulin. (B) The activity of Rac-1 and Cdc42 in primary myoblasts, WJ-MSCs and ESCs. Obtained data is presented as mean ± standard deviation. Student's non-paired t-test was used for statistical analyses. Asterisk marks significant differences ($P < 0.05$).

β-galactosidase positive cells, human WJ-MSCs on the basis of human nuclear antigen immunolocalization, and ESCs on the basis of histone H2B-GFP fluorescence. The efficiency of the participation of transplanted cells in the muscle regeneration was assessed on the basis of the number of cells able to home injured muscle and/or to form new muscle fibers. Transplanted primary myoblasts formed new muscle fibers with the highest efficiency, as compared to other cells analyzed (Fig. 7A and B). Control primary myoblasts transplanted to muscles injected with saline participated in the formation of 4.7% +/− 3% muscle fibers. In Sdf-1 treated muscles this proportion reached 8.2% +/− 3.5%. Finally, Sdf-1-treated myoblasts injected to Sdf-1-injected muscles participated in the formation of 12.1% +/− 5.5% fibers (Fig. 7A and B). Silencing of Cxcr4 expression significantly decreased the number of myofibers formed with the participation of transplanted myoblasts (Fig. 7B). Silencing of Cxcr7 expression did not significantly change the number of myofibers formed with the participation of transplanted myoblasts (Fig. 7B). Thus, co-injection of myoblasts and Sdf-1 improved participation of myoblast in formation of new myofibers.

The WJ-MSCs transplanted into injured muscles, control or Sdf-1 injected, only very rarely were found within regenerating tissue. Only few of them were able to participate in formation of new muscle fibers (data not shown). On the other hand, ESCs were able to home regenerating tissue and were easily detectable between muscle fibers. However, these cells also only very rarely were found to participate in the formation of muscle fibers. In control, saline-injected muscles most of the control, untreated ESCs formed aggregates surrounded with muscle fiber basal lamina. Only single cells were localized along basal lamina. The localization of ESCs changed when muscles were injected with Sdf-1. Under such conditions ESCs were able to migrate and localize along muscle fiber basal lamina. In this case they very

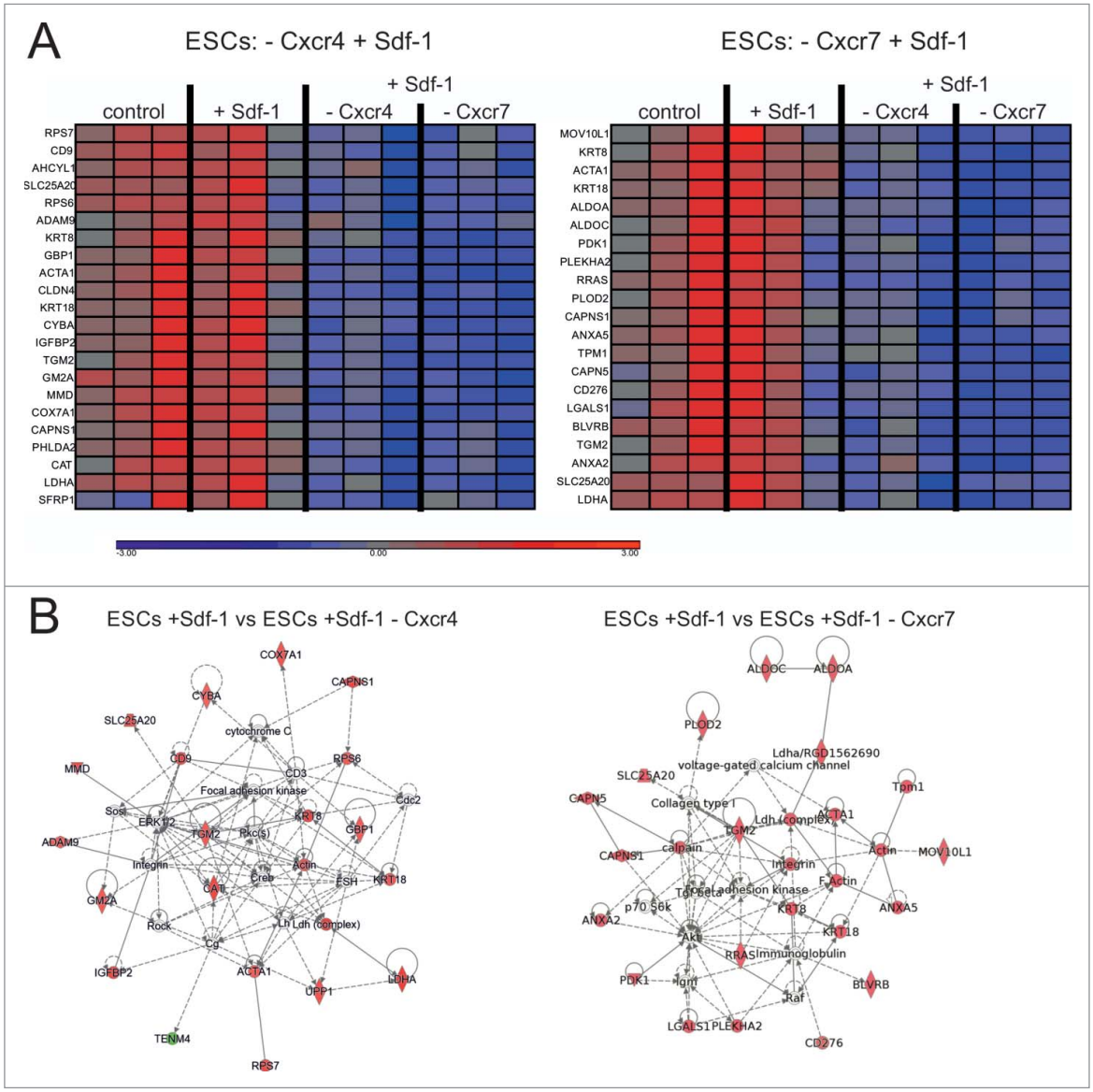

Figure 5. Sdf-1 impact at global gene expression in *in vitro* cultured ESCs. (A) Transcription profile of genes in ESCs. Blue color indicates low and red color indicates high expression levels of mRNA transcripts. (B) Gene networks created by interposing the results onto database of Ingenuity containing information about the gene function with the use of Ingenuity Pathway Analysis tool.

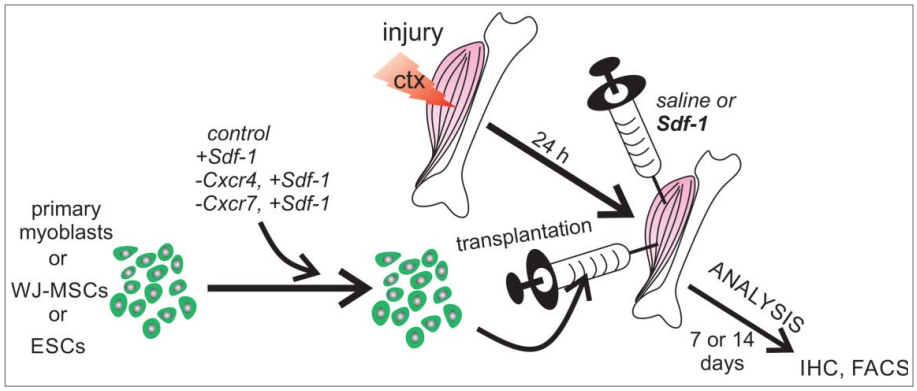

Figure 6. The experimental design of *in vivo* analyses.

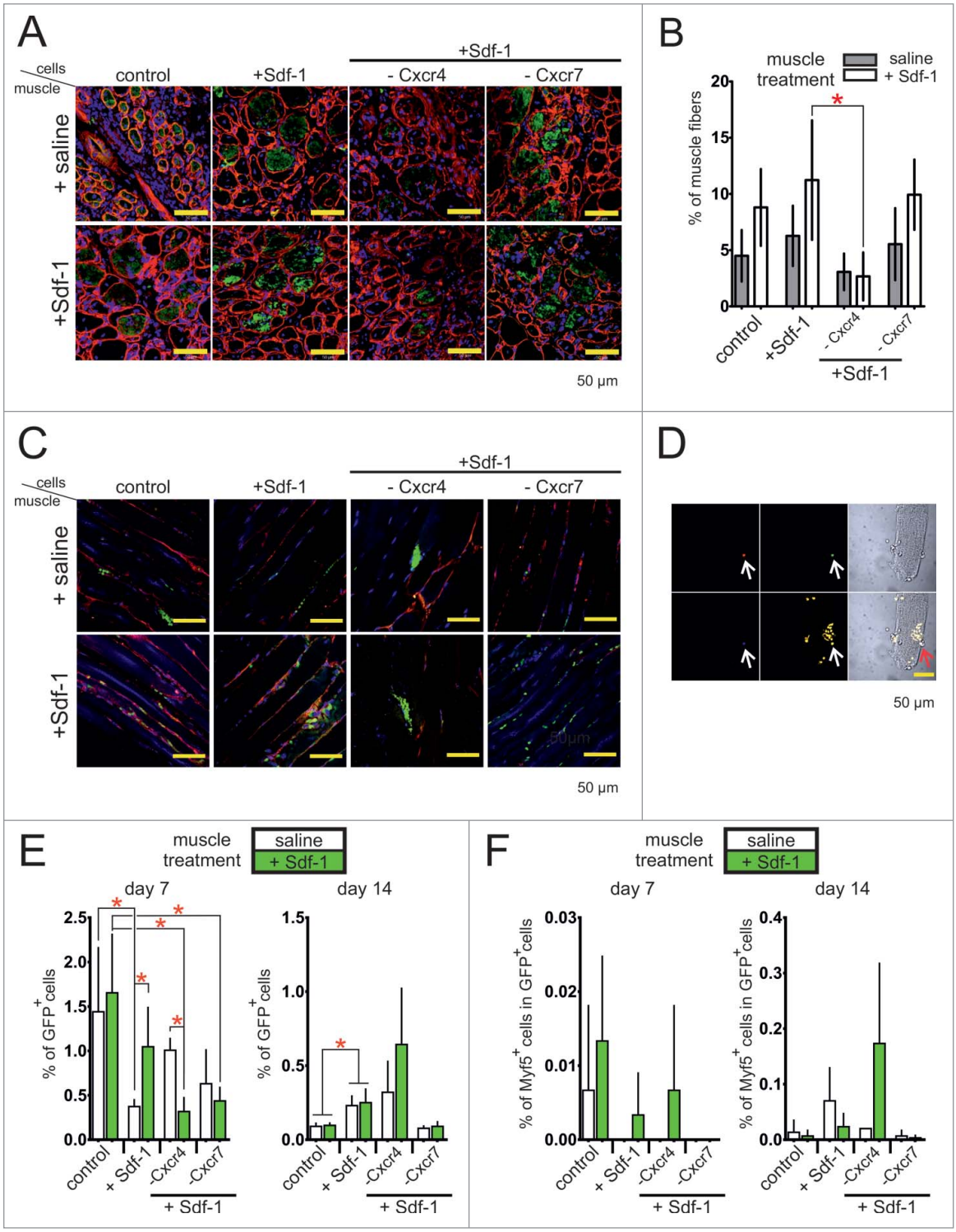

Figure 7. The localization of primary myoblasts and ESCs after transplantation to injured gastrocnemius muscle. (A) The localization of transplanted primary myoblasts expressing β-galactosidase in cross section of muscle at day 7 of regeneration (green - β-galactosidase, blue – chromatin, red – immunolocalization of laminin). (B) The proportion of muscle fibers formed with the participation of transplanted myoblasts in cross sections of muscle at day 7 of regeneration (n = 5). (C) The localization of transplanted ESCs expressing Green Fluoresent protein (GFP) in longitudinal section of muscle at day 7 of regeneration (green - GFP, blue – chromatin, red – immunolocalization of laminin). (D) The localization of mononucleated cells at muscle fiber isolated from skeletal muscle engrafted with ESCs expressing GFP analyzed at day 7 of regeneration (green – GFP, red – immunolocalization of GFP using anti-GFP antibody, blue – immunolocalization of Myod1, yellow - chromatin). (E) Proportion of ESCs expressing GFP in the population of mononucleated cells isolated from the muscle at day 7 and 14 of regeneration (n = 3). (F) Proportion of ESCs expressing Myf5 in the population of GFP expressing ESCs (n = 3). FACS analysis of results is presented as mean ± standard deviation. Student's non-paired t-test was used for statistical analyses. Asterisk marks significant differences ($P < 0.05$).

rarely formed aggregates and were mostly visible as a single cells (Fig. 7C). Similar behavior characterized Sdf-1 treated ESCs transplanted either into control or Sdf-1 treated muscles. Silencing of Cxcr4 but not Cxcx7 expression led to the decrease of ESCs migration. As a result transplanted cells were localized mostly in aggregates. FACS analysis allowed us to verify the proportion of ESCs present within the muscle at days 7 and 14 of regeneration. Generally, in the population of mononucleated cells isolated from the regenerating muscle we were able to detect between 0.32% and 1.65% of ESCs at day 7 and only 0.08% – 0.64% of ESCs at day 14 (Fig. 7E). ESCs were identified on the basis of histone H2B-GFP fluorescence. At day 7 of regeneration the proportion of ESCs detectable within the muscles injected with saline was 0.37%–1.44% and it reached 0.32%–1.65% in the muscles treated with Sdf-1. At day 14 of regeneration the proportion of ESCs was very low (less than 0.64%) and it was comparable between control and Sdf-1 treated muscles. Silencing of Cxcr4 or Cxcr7 expression did not decreased the proportion of ESCs present in regenerating muscles (Fig. 7E). Regardless of their localization, ESCs cells very rarely expressed myogenic transcription factors, such as Myod1, as demonstrated by immunolocalization (Fig. 7D). At day 7 of regeneration up to 0.01% of GFP positive cells i.e., ESCs isolated from the muscles expressed Myf5, as shown by FACS analysis (Fig. 7F). At day 14 of regeneration the proportion of Myf5 expressing ESCs reached 0.17%. Sdf-1 did not change this proportion. Summarizing, Sdf-1 improved the ability of ESCs to migrate in injured muscle. However, these cells only very rarely initiated myogenic differentiation when transplanted into injured muscle.

Discussion

Our study shows that Sdf-1, acting via Cxcr4, increased primary myoblast, WJ-MSC, and ESCs ability to migrate *in vitro*. Except increasing the expression of CD9[24] Sdf-1 also impacts at the expression and activation of other proteins engaged in cell adhesion and migration. Sdf-1 treatment also resulted in the activation of FAK, i.e. non-receptor tyrosine kinase present in focal contacts composed of proteins anchoring integrins with actin cytoskeleton (reviewed in ref. 40). Sdf-1 dependent FAK activation could be achieved by stimulating both Cxcr4 and Cxcr7 receptors. Active FAK kinase is a key component of many signal transduction pathways (reviewed in ref. 43). However, from our point of view, the role of FAK in the activation of cell motility is the most important one (reviewed in ref. 44). Among such functions of FAK could be its positive impact at the MMP-2 and -9 (matrix metalloproteinases-2 and -9) expression and activity influencing extracellular matrix degradation during cells migration.[45,46] Next, FAK signaling controls the formation and turnover of focal contacts[46] and also activates Rho GTPases leading to actin stress fiber formation.[47] In our study, Sdf-1 treatment of cells led also to the activation of GTPases: Rac-1 and Cdc42 belonging to Rho GTPases family. Importantly, activation of these proteins depended only at Cxcr4. It was shown previously that Rac-1 mediates actin polymerization in lamellipodia at the front of migrating cells and Cdc42 induced actin polymerization in filopodia and invadopodia.[48]

Our study also reveals the differences in cell signaling mediated by Sdf-1 - Cxcr4 and Cxcr7 pathways. Cxcr4 interacts with Sdf-1 but Cxcr7 except Sdf-1 also binds chemokine I-TAC (CXCL11) (reviewed in ref. 49). By silencing each of these receptors we were able to distinguish which one is involved in the regulation of certain genes. Thus, in ESCs expression of CD9 is regulated via Sdf-1 activating Cxcr4, but not Cxcr7, what was in agreement with our previous results documenting Sdf-1 dependent expression of CD9 in C2C12 myoblasts, bone marrow derived MSC, and ESCs.[24] Here we showed that also expression of ADAM9 is induced in ESCs by Sdf-1 in Cxcr4 dependent manner. The role of ADAM-9 in the cell migration was previously documented for keratinocytes and fibroblasts.[50,51] Analysis of keratinocytes showed that ADAM-9 regulates cells migration by interaction with integrin $\beta 1$ and regulation of MMPs synthesis.[50] Thus, activation of FAK, Rac-1, and Cdc42, as well as induction of CD9 and ADAM-9 expression, underlay the ability of studied cells to migrate.

We show that Sdf-1, acting *via* Cxcr4, increased myoblast ability to migrate *in vitro* and participate in the formation of new muscle fibers *in vivo* when transplanted intramuscularly. The effect of Sdf-1 treatment was manifested better when myoblasts and Sdf-1 were co-injected than when myoblast were pre-treated with Sdf-1. On the other hand, it was shown that the pre-incubation of myoblasts with bFGF or Concanavalin A was shown to increase the efficiency of myoblasts transplantation.[52-54] The effect we observed was also similar to that documented for other cell types, such as mesoangioblasts, which pre-treatment with Sdf-1 or tumor necrosis factor α (TNF-α) enhanced their delivery and led to a complete reconstitution of skeletal muscles in mice that serve as a mouse model of severe muscular dystrophy.[55] In our hands the pre-treatment of ESCs with Sdf-1 or co-injection of ESCs and Sdf-1 into skeletal muscles increased their ability to migrate within the regenerating tissue. Previously, we showed that ESCs pretreatment improved the ability of ESCs to migrate and fuse with myoblasts *in vitro*.[24] Since, Sdf-1 increased the expression of CD9 in ESCs we postulated that it might facilitate the fusion.[24] Currently, we also documented that Sdf-1 promoted migration of ESCs within injured muscle and stimulated these cells to align in the manner characteristic for fusing myoblasts. Unfortunately, it did not affect the ESCs ability to initiate myogenic differentiation and fusion with myoblasts *in vivo*. However, it was

previously shown that in order to induce myogenic differentiation of ESCs one has to either overexpress myogenic factors such as MyoD, Pax3 or Pax7 or treat the them with precisely designed cocktail of factors (reviewed in ref. 33). ESCs that were not subjected to such treatments fail to effectively differentiate and fuse with myoblasts most probably due to the fact that they do not initiate the expression of M-cadherin or vascular cell adhesion molecule (V-CAM1) that are also crucial for fusion.[25]

MSCs isolated from Wharton jelly (WJ-MSCs), as well as adherent fraction of human umbilical cord blood cells, i.e., the cells that constitute the subpopulation enriched in MSCs, were shown by us to be able to follow myogenic program both *in vitro* and *in vivo*.[26,57] In our hands WJ-MSCs were able to colonize injured skeletal muscle and, with frequency of 5.3%, participate in the formation of new muscle fibers. Pre-treatment of WJ-MSCs with Sdf-1 did not impact their ability to form new muscle fibers but significantly increased muscle mass. Interestingly, *in vitro* these cells manifested myogenic potential and formed hybrid myotubes with C2C12 myoblasts.[26] Currently we documented that Sdf-1 treatment induced migration of WJ-MSC *in vitro*. However, this stimulation was not sufficient to improve their participation in the muscle reconstruction. Our result is in bright contrast to other study which showed that MSCs isolated from rat or human bone marrow participated in the formation of as many as 60–70% of new muscle fibers and restored expression of dystrophin in *mdx* mice muscles.[57] Thus, MSCs isolated form varied sources could differ in their myogenic potential.

Summarizing, Sdf-1 improved the ability of primary myoblasts and ESCs to migrate within the injured muscle. Moreover, injected intramuscularly Sdf-1 stimulated the transplanted primary myoblasts to participate in the formation of new muscle fibers. Mechanisms controlling cells migration activated by Sdf-1 rely at Cxcr4-dependent signaling pathways leading to the activation of proteins engaged in the focal contacts formation and actin polymerization, such as FAK, Rac-1, and Cdc42, as well as the expression of CD9 and ADAM-9. Sdf-1 - Cxcr7 interactions change the expression and activation of proteins engaged in cell migration, however, these changes does not result in the alternation of cell motility. Sdf-1 certainly improves migration of transplanted cells, however, fails to efficiently induce their myogenic differentiation. However, one has to remember that, as we shown previously, it greatly impacts at the homing of endogenous stem cells and by doing that improves muscle regeneration.[23,57]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

[1] Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 2012; 139:2845-56; PMID:22833472; https://doi.org/10.1242/dev.069088

[2] Gibson MC, Schultz E. Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 1983; 6:574-80; PMID:6646160; https://doi.org/10.1002/mus.880060807

[3] Barani AE, Durieux AC, Sabido O, Freyssenet D. Age-related changes in the mitotic and metabolic characteristics of muscle-derived cells. J Appl Physiol 2003; 95:2089-98; PMID:14555672; https://doi.org/10.1152/japplphysiol.00437.2003

[4] Kharraz Y, Guerra J, Pessina P, Serrano AL, Munoz-Canoves P. Understanding the Process of Fibrosis in Duchenne Muscular Dystrophy. BioMed Res Int 2014; 2014:965631; PMID:24877152; https://doi.org/10.1155/2014/965631

[5] Sirabella D, De Angelis L, Berghella L. Sources for skeletal muscle repair: from satellite cells to reprogramming. J Cachexia Sarcopenia Muscle 2013; 4:125-36; PMID:23314905; https://doi.org/10.1007/s13539-012-0098-y

[6] Bajek A, Porowinska D, Kloskowski T, Brzoska E, Ciemerych MA, Drewa T. Cell therapy in Duchenne Muscular Dystrophy treatment. Crit Rev Eukaryot Gene Expr 2015; 25:1-11; PMID:25955813; https://doi.org/10.1615/CritRevEukaryotGeneExpr.2015011074

[7] Briggs D, Morgan JE. Recent progress in satellite cell/myoblast engraftment – relevance for therapy. FEBS J 2013; 280:4281-93; PMID:23560812; https://doi.org/10.1111/febs.12273

[8] Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature 1989; 337:176-9; PMID:2643055; https://doi.org/10.1038/337176a0

[9] Cerletti M, Jurga S, Witczak CA, Hirshman MF, Shadrach JL, Goodyear LJ, Wagers AJ. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell 2008; 134:37-47; PMID:18614009; https://doi.org/10.1016/j.cell.2008.05.049

[10] Xu X, Wilschut KJ, Kouklis G, Tian H, Hesse R, Garland C, Sbitany H, Hansen S, Seth R, Knott PD, et al. Human satellite cell transplantation and regeneration from diverse skeletal muscles. Stem Cell Rep 2015; 5:419-34; PMID:26352798; https://doi.org/10.1016/j.stemcr.2015.07.016

[11] Costamagna D, Berardi E, Ceccarelli G, Sampaolesi M. Adult stem cells and skeletal muscle regeneration. Curr Gene Ther 2015; 15:348-63; PMID:26122100; https://doi.org/10.2174/1566523215666150630121024

[12] Gussoni E, Pavlath GK, Lanctot AM, Sharma KR, Miller RG, Steinman L, Blau HM. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. Nature 1992; 356:435-8; PMID:1557125; https://doi.org/10.1038/356435a0

[13] Huard J, Roy R, Bouchard JP, Malouin F, Richards CL, Tremblay JP. Human myoblast transplantation between immunohistocompatible donors and recipients produces immune reactions. Transplant Proc 1992; 24:3049-51; PMID:1466052

[14] Huard J, Bouchard JP, Roy R, Malouin F, Dansereau G, Labrecque C, Albert N, Richards CL, Lemieux B, Tremblay JP. Human myoblast transplantation: preliminary results of 4 cases. Muscle Nerve 1992; 15:550-60; PMID:1584246; https://doi.org/10.1002/mus.880150504

[15] Palmieri B, Tremblay JP, Daniele L. Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy. Pediat Transplant 2010; 14:813-9; PMID:20963914; https://doi.org/10.1111/j.1399-3046.2010.01377.x

[16] Partridge T. The current status of myoblast transfer. Neurol Sci 2000; 21:S939-42; PMID:11382193; https://doi.org/10.1007/s100720070007

[17] El Fahime E, Torrente Y, Caron NJ, Bresolin MD, Tremblay JP. *in vivo* migration of transplanted myoblasts requires matrix metalloproteinase activity. Exp Cell Res 2000; 258:279-87; PMID:10896779; https://doi.org/10.1006/excr.2000.4962

[18] Quenneville SP, Chapdelaine P, Skuk D, Paradis M, Goulet M, Rousseau J, Xiao X, Garcia L, Tremblay JP. Autologous transplantation of muscle precursor cells modified with a lentivirus for muscular dystrophy: human cells and primate models. Mol Ther 2007; 15:431-8; PMID:17235323; https://doi.org/10.1038/sj.mt.6300047

[19] Skuk D, Goulet M, Tremblay JP. Use of repeating dispensers to increase the efficiency of the intramuscular myogenic cell injection procedure. Cell Transplant 2006; 15:659-63; PMID:17176617; https://doi.org/10.3727/000000006783981648

[20] Skuk D, Goulet M, Roy B, Tremblay JP. Efficacy of myoblast transplantation in nonhuman primates following simple intramuscular cell injections: toward defining strategies applicable to humans. Exp Neurol 2002; 175:112-26; PMID:12009764; https://doi.org/10.1006/exnr.2002.7899

[21] Skuk D, Goulet M, Tremblay JP. Transplanted myoblasts can migrate several millimeters to fuse with damaged myofibers in nonhuman primate skeletal muscle. J Neuropathol Exp Neurol 2011; 70:770-8; PMID:21865885; https://doi.org/10.1097/NEN.0b013e31822a6baa

[22] Lafreniere JF, Caron MC, Skuk D, Goulet M, Cheikh AR, Tremblay JP. Growth factor coinjection improves the migration potential of monkey myogenic precursors without affecting cell transplantation success. Cell Transplant 2009; 18:719-30; PMID:19523340; https://doi.org/10.3727/096368909X470900

[23] Brzoska E, Kowalewska M, Markowska-Zagrajek A, Kowalski K, Archacka K, Zimowska M, Grabowska I, Czerwinska AM, Czarnecka-Gora M, Streminska W, et al. Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. Biol Cell 2012; 104:722-37; PMID:22978573; https://doi.org/10.1111/boc.201200022

[24] Brzoska E, Kowalski K, Markowska-Zagrajek A, Kowalewska M, Archacki R, Plaskota I, Streminska W, Janczyk-Ilach K, Ciemerych MA. Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration. Stem Cell Res Ther 2015; 6:46; PMID:25890097; https://doi.org/10.1186/s13287-015-0041-1

[25] Archacka K, Denkis A, Brzoska E, Swierczek B, Tarczyluk M, Janczyk-Ilach K, Ciemerych MA, Moraczewski J. Competence of *in vitro* cultured mouse embryonic stem cells for myogenic differentiation and fusion with myoblasts. Stem Cells Dev 2014; 23:2455-68; PMID:24940624; https://doi.org/10.1089/scd.2013.0582

[26] Grabowska I, Brzoska E, Gawrysiak A, Streminska W, Moraczewski J, Polanski Z, Hoser G, Kawiak J, Machaj EK, Pojda Z, et al. Restricted myogenic potential of mesenchymal stromal cells isolated from umbilical cord. Cell Transplant 2012; 21:1711-26; PMID:22525423; https://doi.org/10.3727/096368912X640493

[27] Farini A, Sitzia C, Erratico S, Meregalli M, Torrente Y. Clinical applications of mesenchymal stem cells in chronic diseases. Stem Cells Int 2014; 2014:306573; PMID:24876848; https://doi.org/10.1155/2014/306573

[28] Moroni L, Fornasari PM. Human mesenchymal stem cells: a bank perspective on the isolation, characterization and potential of alternative sources for the regeneration of musculoskeletal tissues. J Cell Physiol 2013; 228:680-7; PMID:22949310; https://doi.org/10.1002/jcp.24223

[29] Meyer S, Yarom R. Muscle regeneration and transplantation enhanced by bone marrow cells. Br J Exp Pathol 1983; 64:15-24; PMID:6340712

[30] Shi D, Reinecke H, Murry CE, Torok-Storb B. Myogenic fusion of human bone marrow stromal cells, but not hematopoietic cells. Blood 2004; 104:290-4; PMID:15010375; https://doi.org/10.1182/blood-2003-03-0688

[31] Leroux L, Descamps B, Tojais NF, Seguy B, Oses P, Moreau C, Daret D, Ivanovic Z, Boiron JM, Lamaziere JM, et al. Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. Mol Ther 2010; 18:1545-52; PMID:20551912; https://doi.org/10.1038/mt.2010.108

[32] Grabowska I, Archacka K, Czerwinska AM, Krupa M, Ciemerych MA. Mouse and human pluripotent stem cells and the means of their myogenic differentiation. Results Probl Cell Differ 2012; 55:321-56; PMID:22918815; https://doi.org/10.1007/978-3-642-30406-4_18

[33] Swierczek B, Ciemerych MA, Archacka K. From pluripotency to myogenesis: a multistep process in the dish. J Muscle Res Cell Motil 2015; PMID:26715014

[34] Chal J, Oginuma M, Al Tanoury Z, Gobert B, Sumara O, Hick A, Bousson F, Zidouni Y, Mursch C, Moncuquet P, et al. Differentiation of pluripotent stem cells to muscle

fiber to model Duchenne muscular dystrophy. Nat Biotechnol 2015; 33:962-9; PMID:26237517; https://doi.org/10.1038/nbt.3297

[35] Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. *in vitro* Cell Dev Biol Anim 1995; 31:773-9; PMID:8564066

[36] Hadjantonakis AK, Macmaster S, Nagy A. Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. BMC Biotechnol 2002; 2:11; PMID:12079497; https://doi.org/10.1186/1472-6750-2-11

[37] Goetsch KP, Niesler CU. Optimization of the scratch assay for *in vitro* skeletal muscle wound healing analysis. Anal Biochem 2011; 411:158-60; PMID:21146491; https://doi.org/10.1016/j.ab.2010.12.012

[38] Eisenhart C. The assumptions underlying the analysis of variance. Biometrics 1947; 3:1-21; PMID:20240414; https://doi.org/10.2307/3001534

[39] Heiberger RM, Holland B. Statistical analysis and data display: an intermediate course with examples in R. Springer-Verlag New York; 2015.

[40] Graham ZA, Gallagher PM, Cardozo CP. Focal adhesion kinase and its role in skeletal muscle. J Muscle Res Cell Motil 2015; 36:305-15; PMID:26142360

[41] Lee JG, Heur M. Interleukin-1beta-induced Wnt5a enhances human corneal endothelial cell migration through regulation of Cdc42 and RhoA. Mol Cell Biol 2014; 34:3535-45; PMID:25022753; https://doi.org/10.1128/MCB.01572-13

[42] Sadok A, Marshall CJ. Rho GTPases: masters of cell migration. Small GTPases 2014; 5:e29710; PMID:24978113; https://doi.org/10.4161/sgtp.29710

[43] Guan JL. Role of focal adhesion kinase in integrin signaling. Int J Biochem Cell Biol 1997; 29:1085-96; PMID:9416004; https://doi.org/10.1016/S1357-2725(97)00051-4

[44] Schlaepfer DD, Mitra SK. Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev 2004; 14:92-101; PMID:15108811; https://doi.org/10.1016/j.gde.2003.12.002

[45] Zhang Y, Thant AA, Hiraiwa Y, Naito Y, Sein TT, Sohara Y, Matsuda S, Hamaguchi M. A role for focal adhesion kinase in hyluronan-dependent MMP-2 secretion in a human small-cell lung carcinoma cell line, QG90. Biochem Biophys Res Commun 2002; 290:1123-7; PMID:11798192; https://doi.org/10.1006/bbrc.2001.6321

[46] Hsia DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, et al. Differential regulation of cell motility and invasion by FAK. J Cell Biol 2003; 160:753-67; PMID:12615911; https://doi.org/10.1083/jcb.200212114

[47] Schaller MD. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 2001; 1540:1-21; PMID:11476890; https://doi.org/10.1016/S0167-4889(01)00123-9

[48] Ridley AJ. Rho GTPase signalling in cell migration. Curr Opin Cell Biol 2015; 36:103-12; PMID:26363959; https://doi.org/10.1016/j.ceb.2015.08.005

[49] Sanchez-Martin L, Sanchez-Mateos P, Cabanas C. CXCR7 impact on CXCL12 biology and disease. Trends Mol Med 2013; 19:12-22; PMID:23153575; https://doi.org/10.1016/j.molmed.2012.10.004

[50] Zigrino P, Steiger J, Fox JW, Loffek S, Schild A, Nischt R, Mauch C. Role of ADAM-9 disintegrin-cysteine-rich domains in human keratinocyte migration. J Biol Chem 2007; 282:30785-93; PMID:17704059; https://doi.org/10.1074/jbc.M701658200

[51] Nath D, Slocombe PM, Webster A, Stephens PE, Docherty AJ, Murphy G. Meltrin gamma(ADAM-9) mediates cellular adhesion through alpha(6)beta(1)integrin, leading to a marked induction of fibroblast cell motility. J Cell Sci 2000; 113(Pt 12):2319-28; PMID:10825303

[52] Kinoshita I, Vilquin JT, Tremblay JP. Pretreatment of myoblast cultures with basic fibroblast growth factor increases the efficacy of their transplantation in mdx mice. Muscle Nerve 1995; 18:834-41; PMID:7630343; https://doi.org/10.1002/mus.880180806

[53] Kinoshita I, Vilquin JT, Roy R, Tremblay JP. Successive injections in mdx mice of myoblasts grown with bFGF. Neuromuscul Disord 1996; 6:187-93; PMID:8784807; https://doi.org/10.1016/0960-8966(96)00004-1

[54] Ito H, Hallauer PL, Hastings KE, Tremblay JP. Prior culture with concanavalin A increases intramuscular migration of transplanted myoblast. Muscle Nerve 1998; 21:291-7; PMID:9486857; https://doi.org/10.1002/(SICI)1097-4598(199803)21:3%3c291::AID-MUS2%3e3.0.CO;2-5

[55] Galvez BG, Sampaolesi M, Brunelli S, Covarello D, Gavina M, Rossi B, Constantin G, Torrente Y, Cossu G. Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. J Cell Biol 2006; 174:231-43; PMID:16831885; https://doi.org/10.1083/jcb.200512085

[56] Grabowska I, Streminska W, Janczyk-Ilach K, Machaj EK, Pojda Z, Hoser G, Kawiak J, Moraczewski J, Ciemerych MA, Brzoska E. Myogenic potential of mesenchymal stem cells - the case of adhesive fraction of human umbilical cord blood cells. Curr Stem Cell Res Ther 2013; 8:82-90; PMID:23270632; https://doi.org/10.2174/1574888X11308010010

[57] Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S, Ide C, Nabeshima Y. Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science 2005; 309:314-7; PMID:16002622; https://doi.org/10.1126/science.1110364

[58] Kowalski K, Archacki R, Archacka K, Stremińska W, Paciorek A, Gołąbek M, Ciemerych MA, Brzoska E. Stromal derived factor-1 and granulocyte-colony stimulating factor treatment improves regeneration of Pax7−/− mice skeletal muscles. J Cachexia Sarcopenia Muscle 2015; PMID:27239402

Pozostałe publikacje, rozdziały w książkach, źródła finansowania, stypendia, wyróżnienia

Publikacje i rozdziały w książkach niewchodzące w skład rozprawy doktorskiej.

Publikacje oryginalne:

Edyta Brzoska, Magdalena Kowalewska, Agnieszka Markowska, **Kamil Kowalski**, Karolina Archacka, Małgorzata Zimowska, Iwona Grabowska, Areta M Czerwińska, Magdalena Czarnecka-Góra, Władysława Stremińska, Katarzyna Jańczyk-Ilach, Maria A Ciemerych *Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells*. BIOLOGY OF THE CELL, 2012; 104(12).

Iwona Grabowska, Magdalena A. Mazur, **Kamil Kowalski**, Anita Helinska, Jerzy Moraczewski, Władysława Stremińska, Grażyna Hoser, Jerzy Kawiak, Maria A. Ciemerych, Edyta Brzoska:
Progression of inflammation during immunodeficient mouse skeletal muscle regeneration. JOURNAL OF MUSCLE RESEARCH AND CELL MOTILITY, 2015; 36(6).

Publikacje przeglądowe:

Karolina Archacka, **Kamil Kowalski**, Edyta Brzóska:
Czy komórki satelitowe są macierzyste? POSTEPY BIOCHEMII, 2013; 59(2).

Kamil Kowalski, Agnieszka Markowska-Zagrajek, Izabela Plaskota, Magdalena Kowalewska, Rafał Archacki, Karolina Archacka, Areta Czerwinska, Iwona Grabowska, Małgorzata Zimowska, Katrzyna Jańczyk-Ilach, Władysława Stremińska, Maria A. Ciemerych, Edyta Brzóska-Wójtowicz:
Rola SDF-1 w procesach regeneracji i nowotworzenia. POSTĘPY POLSKIEJ MEDYCYNY I FARMACJI, 2012; 2(1)

Rozdział w książce:

Karolina Archacka, Edyta Brzoska, Maria A. Ciemerych, Areta M. Czerwinska, Iwona Grabowska, **Kamil K. Kowalski**, Malgorzata Zimowska.
Pluripotent and Mesenchymal Stem Cells—Challenging Sources for Derivation of Myoblast. CARDIAC CELL CULTURE TECHNOLOGIES, 2018: pages 109-154;

Projekty naukowe:

2016-2019
„Wykorzystanie potencjału regeneracyjnego mezenchymalnych komórek macierzystych”
STRATEGMED, EXPLORE ME, Narodowe Centrum Badań i Rozwoju.
Lider: Instytut Medycyny Doświadczalnej i Klinicznej, koordynator: dr hab. Mirosław Janowski,
wykonawca projektu

2016-2017
„Różnicowanie komórek macierzystych szpiku kostnego w niszy komórek satelitowych, analiza funkcjonalna zróżnicowanych komórek”
Dotacja celowana na prowadzenie badań naukowych dla młodych naukowców, Wydział Biologii, 501-D114-86-0115
kierownik projektu

2015-2016
„Rola Sdf-1 w migracji i różnicowaniu komórek macierzystych o potencjale miogenicznym-różnicowanie komórek macierzystych szpiku kostnego w niszy komórek satelitowych”
Dotacja celowana na prowadzenie badań naukowych dla młodych naukowców, Wydział Biologii, 501/86-118617
kierownik projektu

2014 2017:
„Znaczenie Sdf-1 i jego receptora Cxcr7 we wczesnych etapach różnicowania zarodkowych komórek macierzystych”
PRELUDIUM 11, Narodowe Centrum Nauki, 2013/11/N/NZ3/00186
kierownik projektu

2014-2015:
„Rola Sdf-1 w migracji komórek macierzystych pod błonę podstawną włókna mięśniowego”
Dotacja celowana na prowadzenie badań naukowych dla młodych naukowców, Wydział Biologii, 501/86-107423,
kierownik projektu

2014-2017:
„Molekularne mechanizmy i markery progresji płaskonabłonkowego raka sromu”
SONATA BIS 3, Narodowe Centrum Nauki, 2013/10/E/NZ5/00663,
Kierownik dr hab. Magdalena Kowalewska, Warszawski Uniwersytet Medyczny,
wykonawca projektu

2013- 2015:
„Participation of stem cells in muscle regeneration - cell signaling pathways”
POMOST, Fundacja na rzecz Nauki Polskiej, Pomost/2011-4/3,
Kierownik: dr hab. Edyta Brzóska-Wójtowicz,
wykonawca/stypendysta

2012-2013:
„Wpływ Sdf-1 na zdolność komórek macierzystych do fuzji. Określenie roli białka CD9”
Dotacja celowana na prowadzenie badań naukowych dla młodych naukowców, Wydział Biologii, 501/86-104920,
kierownik projektu

2012-2014
„*Aktywacja mobilizacji komórek macierzystych ze szpiku kostnego do regenerujących mięśni”*
IUVENTUS PLUS, MNiSW, IP2011 004871,
Kierownik: dr hab. Edyta Brzóska-Wójtowicz,
wykonawca projektu

2012
„Stymulacja regeneracji mięśni szkieletowych - potencjalna rola cytokiny SDF-1”
NAUKOWA FUNDACJA POLPHARMY, 2/VII/2008,
Kierownik: dr hab. Edyta Brzóska-Wójtowicz,
wykonawca projektu

Stypendia i nagrody:

2016-2017

Stypendium doktoranckie **ETIUDA** 20, Narodowe Centrum Nauki 2016/20/T/NZ3/00300

„Różnicowanie komórek macierzystych szpiku kostnego w komórki o potencjale miogenicznym, wpływ niszy komórek satelitowych oraz chemokiny Sdf-1”

w tym 3-miesięczny staż zagraniczny. Paryż, Instytut Cochin, Zakład Genetyki, Rozwoju i Fizjopatologii Mięśni Szkieletowych, opiekun Prof. Pascal Maire.

2017-2018

Stypendium **START** Fundacja na rzecz Nauki Polskiej, START 035.2017

2012-2013 i 2015-2016

Nagroda Rektora za Osiągnięcia Naukowe (nagroda zespołowa)

2012-2013 i 2013-2014

Stypendium dla najlepszych doktorantów Wydziału Biologii UW

2013-2014, 2015-2016, 2016-2017

Stypendium doktoranckie wypłacane z dotacji podmiotowej - projakościowej UW

Nagroda specjalna za najlepszą pracę podczas V Ogólnopolskiej Konferencji Naukowej dla lekarzy, młodych naukowców i studentów „Postępy w Badaniach Biomedycznych” Warszawa, 29 listopad 2014, prezentacja, „Sdf-1 wspomaga regenerację mięśni szkieletowych poprzez mobilizację endogennych komórek macierzystych.”

Oświadczenie współautorów publikacji naukowej:

Kowalski K, Archacki R, Archacka K, Streminska W, Paciorek A, Golabek M, Ciemerych M A, Brzoska E., 2016, *Sdf-1 and G-CSF treatment improves regeneration of Pax7-/- mice skeletal muscles*, JOURNAL OF CACHEXIA, SARCOPENIA AND MUSCLE, 7(4), 483-96, doi: 10.1002/jcsm.12092

Oświadczam, że jestem świadomy/świadoma, że powyższa publikacja naukowa wchodzi w skład rozprawy doktorskiej mgr Kamila Kowalskiego. Potwierdzam również mój wkład w powstanie powyższej publikacji:

Kamil Kowalski .....

Rafał Archacki .....

Karolina Archacka .....

Władysława Stremińska .....

Anna Paciorek .....

Magdalena Gołąbek .....

Maria A. Ciemerych .....

Edyta Brzóska .....

Kowalski K. Współtworzenie koncepcji i projektowanie doświadczeń. Prowadzenie hodowli szczepu myszy pozbawionych funkcjonalnego genu Pax7, doświadczenie *in vivo*: uszkadzanie mięśnia i podawanie *in vivo* badanych czynników, izolacja mięśni i próbek krwi. Wykonanie analiz histochemicznych, immunohistochemicznych, Western blott, qPCR, cystometrii przepływowej. Hodowla włókien mięśniowych i komórek ze szpiku kostnego. Opracowanie wyników, przygotowanie figur, udział w przygotowaniu artykułu.

Archacki R. Udział w analizie wyników qPCR.

Archacka K. Udział w zbieraniu próbek.

Stremińska W. Udział w podawanie *in vivo* analizowanych czynników.

Paciorek A. Udział w analizie preparatów.

Golabek M. Udział w analizie preparatów.

Ciemerych M.A. Recenzja manuskryptu.

Brzoska E. Koordynator projektu, stworzenie koncepcji badań. Przygotowanie artykułu i jego korekta.

Oświadczenie współautorów publikacji naukowej:

Brzóska E, Kowalski K, Markowska-Zagrajek A, Kowalewska M, Archacki R, Plaskota I, Stremińska W, Jańczyk-Ilach K, Ciemerych M A., 2015, *Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration*, STEM CELLS RESEARCH & THERAPY, 24;6:46, doi: 10.1186/s13287-015-0041-1

Oświadczam, że jestem świadomy/świadoma, że powyższa publikacja naukowa wchodzi w skład rozprawy doktorskiej mgr Kamila Kowalskiego. Potwierdzam również mój wkład w powstanie powyższej publikacji:

Edyta Brzóska .....

Kamil Kowalski .....

Agnieszka Markowska-Zagrajek .....

Magdalena Kowalewska .....

Rafał Archacki .....

Izabela Plaskota .....

Władysława Stremińska .....

Katarzyna Jańczyk-Ilach .....

Maria A. Ciemerych .....

Brzoska E. Koordynator projektu, stworzenie koncepcji badań. Wykonanie analiz molekularnych i immunocytochemicznych (szczurze komórki satelitowe). Przygotowanie artykułu i jego korekta

Kowalski K. Zaprojektowanie doświadczeń dotyczących ESCs, BMSC oraz myszy Pax7-/-. Wykonanie analiz *in vitro* dotyczących ESCs, BMSC tj. hodowle komórkowe, analiza immunocytochemiczne, kokultura, doświadczenia z wykorzystaniem siRNA, qPCR i Western blott. Analizy *in vivo* dotyczące myszy Pax7-/-, zbieranie próbek i ich analiza tj. immunohistochemia oraz Western blott. Interpretacja i opracowanie wyników, przygotowanie figur, udział w przygotowaniu artykułu.

Markowska-Zagrajek A. Przeprowadzenie analiz molekularnych tj. Western blott i analiza immunohistochemiczna dotycząca mięśni szkieletowych szczura.

Kowalewska M. Udział w analizach molekularnych, analiza wyników.

Archacki R. Udział w analizach molekularnych, analiza wyników.

Plaskota I. Przeprowadzenie analiz molekularnych dotyczących komórek C2C12.

Stremińska W. Zbieranie próbek.

Jańczyk-Ilach K. Zbieranie próbek.

Ciemerych M.A. Recenzja manuskryptu.

Oświadczenie współautorów publikacji naukowej:

Kowalski K, Kołodziejczyk A, Sikorska M, Płaczkiewicz J, Cichosz P, Kowalewska M, Stremińska W, Jańczyk-Ilach K, Koblowska M, Fogtman A, Iwanicka-Nowicka R, Ciemerych MA, and Brzoska E, 2017, *Stem cells migration during skeletal muscle regeneration - the role of Sdf-1/Cxcr4 and Sdf-1/Cxcr7 axis*, CELL ADHESION & MIGRATION, 11(4):384-398, doi: 10.1080/19336918.2016.1227911

Oświadczam, że jestem świadomy/świadoma, że powyższa publikacja naukowa wchodzi w skład rozprawy doktorskiej mgr Kamila Kowalskiego. Potwierdzam również mój wkład w powstanie powyższej publikacji:

Kamil Kowalski	.....
Aleksandra Kołodziejczyk	.....
Maria Sikorska	.....
Jagoda Płaczkiewicz	.....
Paulina Cichosz	.....
Magdalena Kowalewska	.....
Władysława Stremińska	.....
Katarzyna Jańczyk-Ilach	.....
Marta Koblowska	.....
Anna Fogtman	.....
Roksana Iwanicka-Nowicka	.....
Maria A. Ciemerych	.....
Edyta Brzóska	.....

Kowalski K. Współtworzenie koncepcji i projektowanie doświadczeń. Wykonanie analiz in vitro tj. hodowli komórkowych, testów migracji, siRNA, oznaczeń immunocytochemicznych, G-LISA, qPCR, Western blott. Wykonanie doświadczeń in vivo tj. uszkadzanie mięśni szkieletowych, wstrzykiwanie komórek do regenerującego mięśnia, zbieranie próbek, otrzymywanie skrawków mrożeniowych. Analiza uzyskanych wyników i ich interpretacja. Opracowanie wyników, przygotowanie figur, udział w przygotowaniu artykułu.

Kołodziejczyk A. Udział w analizach immunohistochemicznych skrawków mrożeniowych mięśni.

Sikora M. Udział w przygotowaniu i analizie preparatów mięśni szkieletowych nastrzykniętych ESCs.

Płaczkiewicz J. Udział w przygotowaniu i analizie preparatów WJ-MSCs.

Cichosz P. Udział w analizach immunocytochemicznych filamentów aktynowych.

Kowalewska M. Udział w analizach qPCR.

Stremińska W. Udział w podawaniu in vitro komórek.

Jańczyk-Ilach K. Udział w podawaniu in vitro komórek.

Koblowska M. udział w analizach mikromacierzy.

Fogtman A. Udział w analizach mikromacierzy.

Iwanicka-Nowicka R. Udział w analizach mikromacierzy.

Ciemerych M.A. Recenzja manuskryptu.

Brzoska E. Koordynator projektu, stworzenie koncepcji badań. Przygotowanie artykułu i jego korekta.